Universität Bremen

Consequences of quinone-generated oxidative stress on cultured brain astrocytes

Dissertation

Zur Erlangung des Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

des Fachbereichs 2 (Biologie/Chemie)

an der Universität Bremen

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2020

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Datum des Kolloquiums:	21.09.20

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Chapter: 2.2

Exposure of cultured astrocytes to menadione triggers rapid radical formation, glutathione oxidation and Mrp1-mediated export of glutathione disulfide.

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 50 %
Experimental work and/or acquisition of (experimental) data:	ca. 100 %
Data analysis and interpretation:	ca. 80 %
Preparation of Figures and Tables:	ca. 100 %
Drafting of the manuscript:	ca. 100 %

Chapter: 2.3

Beta-lapachone induces severe oxidative stress in rat primary astrocyte cultures that is prevented by the NQO1 inhibitor dicoumarol.

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 33 %
Experimental work and/or acquisition of (experimental) data:	ca. 33 %
Data analysis and interpretation:	ca. 50 %
Preparation of Figures and Tables:	ca. 90 %
Drafting of the manuscript:	ca. 90 %

Date: 22.07.20

Signatures:

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Danksagung

Zuerst möchte ich mich bei Prof. Dr. Ralf Dringen bedanken. Ralf, es war mir eine Freude so lange Teil deiner Arbeitsgruppe sein zu können und von dir lernen zu dürfen. Mir fällt keine Gelegenheit ein, zu der ein Gespräch mit dir nicht mehr Licht ins Dunkel gebracht hätte.

Mein besonderer Dank gilt Prof. Dr. Ursula Rauen und Prof. Dr. Juan P. Bolaños für die Bereitschaft die Gutachten für die vorliegende Arbeit zu erstellen.

Weiterhin möchte ich allen ehemaligen und derzeitigen Mitgliedern der Arbeitsgruppe Dringen mit denen ich Teil der Gruppe sein durfte, meinen tiefen Dank aussprechen für die tolle Zeit. Danke, für die angenehme Arbeitsatmosphäre und für die offenen Bürotüren, egal ob es sich um dringende wissenschaftliche oder um nicht aufzuschiebende private Angelegenheiten handelte.

Meiner Familie, Mama, Papa, Judith und Helga, möchte ich für ihr Verständnis und die Geduld danken, die sie mir entgegengebracht haben. Ich bin nicht immer einfach, aber ich weiß, dass ich mich immer auf euch verlassen kann. Tausend Dank für eure Unterstützung in den letzten Jahren!

Zuletzt möchte ich meiner Verlobten danken. Jana, du gibst mir einen Grund, ohne dich bin ich richtungslos. Du bist das Wichtigste für mich und ich liebe dich.

Summary

Quinones are a variable class of aromatic compounds that are used by cells as essential electron-carrying coenzymes and in medicine as components in drugs. Quinones are potentially hazardous to living cells, as especially their one-electron reduction to a semiquinone is directly connected to the formation of intracellular radicals and oxidative stress, possibly leading to cell death. The enzyme NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) catalyzes the obligatory two-electron reduction of quinones and produces a hydroquinone, circumventing the formation of a semiquinone radical. This mechanism was believed to be generally beneficial for cells but depending on the nature of the formed hydroquinone the originally expected beneficial NQO1 detoxification can in fact lead to toxic bioactivation of certain quinones. For the present thesis the presence of NQO1 in astrocyte primary cultures from rat brain was investigated and the consequences of an application of the quinones and NQO1 substrates menadione and beta-lapachone on the antioxidative glutathione (GSH) metabolism and the vitality of astrocyte primary cultures was elucidated.

Th presence of NQO1 in rat astrocyte cultures was demonstrated by measurement of NQO1 enzymatic activity in lysates from astrocyte cultures. The specific activity of NQO1 was found to be high as compared to literature data of non-cancerous cells and could be almost completely blocked by the NQO1-inhibitor dicoumarol. In addition, presence of NQO1 in astrocyte cultures was confirmed by immunochemical fluorescence staining of astrocyte cultures and by Western blotting of astrocyte lysates for NQO1.

Application of menadione to astrocyte cultures caused fast cellular ROS production and a rapid cellular formation of glutathione disulfide (GSSG) already after 5 min of exposure, as well as a striking loss in total glutathione (GSx) for incubations in the low hour-range. Also, glycolytic lactate production was found weakened and astrocyte membrane integrity was impaired during menadione treatment. The observed GSH oxidation and impairment of vitality were not prevented by the NQO1-inhibitor dicoumarol, leading to the conclusion that a NQO1-independent process triggers the oxidative stress and the observed toxicity of menadione in astrocytes. These data suggest that the chemical reactivity of menadione is the primary cause of GSH oxidation and loss of vitality in astrocyte cultures.

Investigations on the consequences of a beta-lapachone treatment of astrocyte cultures also caused a rapid cellular ROS formation and almost complete oxidation of

the cellular GSH pool within 5 min, were followed by an impairment of lactate release and a loss in membrane integrity of astrocytes. In contrast to menadione, all adverse consequences caused by a treatment of astrocyte cultures with beta-lapachone were prevented by application of the NQO1 inhibitor dicoumarol. This suggests that NQO1 is the primarily responsible enzyme for the toxic bioactivation of beta-lapachone and of the GSSG formation and toxicity observed in astrocyte cultures treated with beta-lapachone.

Tumor cells are described to express high NQO1 activity which led to the application of menadione and beta-lapachone in cancer research and for clinical therapy. As both compounds are small and hydrophobic, they are expected to easily penetrate membranes like the blood-brain barrier. In a perpetual application for cancer therapy brain cells are therefore potentially encountering menadione and beta-lapachone. Under the assumption of rat primary astrocyte cultures as a model system for brain astrocytes it can therefore be speculated that menadione or beta-lapachone could induce severe disturbances in GSH metabolism and vitality of brain astrocytes *in vivo*.

Zusammenfassung

Chinone sind eine variable Klasse von aromatischen Substanzen, die in Zellen als essentielle Elektronen-transportierende Coenzyme dienen und in der Medizin als Komponente in Medikamenten vorhanden sind. Allerdings sind Chinone auch potentiell gefährlich für lebende Zellen. Besonders die Reduktion durch eine Ein-Elektronen Übertragung auf Chinone zum Semichinon ist direkt mit der Bildung von intrazellulären Radikalen und oxidativem Stress verbunden, welche potentiell zum Zelltod führen können. Das Enzym NAD(P)H: Chinon Akzeptor Oxidoreduktase 1 (NQO1) katalysiert die obligatorische Übertragung von zwei Elektronen auf Chinone und bildet dadurch das jeweilige Hydrochinon. Dadurch umgeht die NQO1 die direkte Freisetzung von Semichinon-Radikalen. Anfangs wurde davon ausgegangen, dass dieser enzymatische Mechanismus generell für Zellen protektiv ist. Die tatsächliche Wirkung der Hydrochinon-Bildung durch NQO1 hängt jedoch stark von den chemischen Eigenschaften des produzierten Hydrochinons ab, d.h. die ursprünglich entgiftend geglaubte Aktivität der NQO1 kann tatsächlich auch zur biotoxischen Aktivierung von Chinonen führen. In der vorliegenden Arbeit wurde geprüft, ob NQO1 in primären Astrozytenkulturen von Ratten exprimiert wird. Weiterhin wurden die Konsequenzen einer Inkubation von Astrozyten mit den Chinonen und NQO1 Substraten Menadion oder Beta-lapachon auf den Glutathion Metabolismus und die Vitalität der Zellen analysiert.

Messungen der Enzymaktivität von NQO1 in Lysaten aus Astrozytenkulturen bestätigten die Expression von NQO1 in Astrozyten und die gemessene Aktivität konnte fast vollständig vom NQO1 Inhibitor Dicoumarol blockiert werden. Außerdem konnte festgestellt werden, dass die NQO1 Aktivität in Astrozytenkulturen im Vergleich zu Literaturdaten von NQO1 Aktivitäten in nicht-krebsartigen Zellen hoch ist. Weiterhin wurde die Expression von NQO1 in intakten Astrozytenkulturen durch Antikörper-Detektion und immunochemische Fluoreszenzfärbung in Astrozytenkulturen bestätigt. Zusätzlich konnte die Anwesenheit von NQO1 in Lysaten aus Astrozytenkulturen in Westernblots nachgewiesen werden.

Untersuchungen zu den Folgen einer Inkubation von Astrozyten mit Menadion zeigten eine schnelle zelluläre ROS Produktion und eine unmittelbare Bildung von Glutathiondisulfid (GSSG) bereits nach 5 min, sowie einen starken Verlust an gesamtem Glutathion (GSx) bei Inkubationen von wenigen Stunden. Gleichzeitig wurde die Laktatfreisetzung aus Astrozyten stark verringert und ihre Membranintegrität

beeinträchtigt, wenn sie mit Menadion inkubiert wurden. Die beobachtete Oxidation von GSH und die Toxizität von Menadion konnten nicht vom NQO1 Inhibitor Dicoumarol verhindert werden. Dies lässt darauf schließen, dass NQO1-unabhängige Prozesse der Hauptgrund für die beschriebenen Folgen von Menadion sind. Daher wird vermutet, dass die chemische Reaktivität von Menadion der vorrangige Grund für die GSH Oxidation, den Verlust der Membranintegrität und die geringere Laktatfreisetzung aus Astrozytenkulturen in das Medium ist.

Untersuchungen zu den Folgen einer Inkubation von Astrozyten mit Beta-lapachon zeigten im Ansatz ähnliche Ergebnisse wie für Menadion beschrieben. Schnelle zelluläre ROS Bildung und nahezu komplette Oxidation von zellulärem GSH zu GSSG bereits nach 5 min Inkubationszeit von Astrozyten mit Beta-lapachon wurden gefolgt von einer Verringerung in der Laktatfreisetzung und dem Verlust der Membranintegrität der Astrozyten. Im Gegensatz zu Menadion war allerdings auffallend, dass der NQO1 Inhibitor Dicoumarol die Bildung von GSSG, den Verlust an GSx und das Einsetzen von Toxizität durch Beta-lapachon in Astrozytenkulturen verhinderte. Dies lässt darauf schließen, dass NQO1 das in erster Linie verantwortliche Enzym für die negativen Konsequenzen von Beta-lapachon auf Astrozytenkulturen ist.

In der Literatur ist beschrieben, dass die Aktivität von NQO1 in Tumoren besonders hoch ist. Dies führte dazu, dass Menadion und Beta-lapachone in der Krebsforschung und in der klinischen Therapie erprobt werden. Da sowohl Menadion als auch Beta-lapachon kleine lipophile Moleküle sind, kann davon ausgegangen werden, dass sie die Bluthirnschranke überwinden können. Im Zuge einer andauernden Behandlung mit einem der beiden Chinone kann es also durchaus dazu kommen, dass Gehirnzellen mit Menadion oder Beta-lapachon konfrontiert werden. Unter der Annahme, dass primäre Astrozytenkulturen aus Ratten ein adäquates Testsystem für Astrozyten im intakten Gehirn darstellen, muss daher davon ausgegangen werden, dass sowohl Menadion als auch Beta-lapachon zu erheblichen Veränderungen im GSH Metabolismus und der Vitalität von Astrozyten führen können.

Abbreviations

ε	extinction coefficient
%	percent
°C	degree Celsius
µg	microgram
µL	microliter
µm	micrometer
ANOVA	analysis of variance
ARE	antioxidative response element
ARQ	beta-lapachone analogue
ATP	adenosine triphosphate
BSA	bovine serum albumin
cm	centimeter
CNS	central nervous system
CYP	cytochrome P450
Da	Dalton
DAPI	4′,6-diamidino-2-phenylindole
DCF	dichlorofluorescein
$DCFH_2$-DA	dihydrodichlorofluorescein diacetate
DHR	dihydro rhodamine 123
DME	drug metabolizing enzymes
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
e.g.	for example (Latin: *exempli gratia*)
EC_{50}	half-maximal effective concentration
et al.	and others (Latin: *et alii*)
FAD	flavin adenine dinucleotide (oxidized)
$FADH_2$	flavin adenine dinucleotide (reduced)
FCS	fetal calf serum
g	gravitational acceleration
GFAP	glial fibrillary acidic protein
GLUT	glucose transporter
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione

GSSG	glutathione disulfide
GST	glutathione-*S*-transferase
GSx	total glutathione
h	hour(s)
H33342	HOECHST 33342
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanosulfonic acid
i.e.	that is (Latin: *id est*)
IB	incubation buffer
kDa	kilo Dalton
Keap1	kelch-like ECH-associated protein 1
K_{ic}	inhibitor constant (competitive)
LDH	lactate dehydrogenase
mA	milliampere
MCT	monocarboxylate transporter
mg	milligram
min	minute(s)
mL	milliliter
mM	millimolar (mmol/L)
mmol	millimoles
mRNA	messenger RNA
Mrp1	multidrug resistance protein 1
MTT	dimethyl thiazolyl diphenyl tetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nm	nanometer
nM	nanomolar (nmol/L)
nmol	nanomoles
NQO1	NAD(P)H: quinone acceptor oxidoreductase 1
NQO2	NRH: quinone acceptor oxidoreductase 2
Nrf2	nuclear factor erythroid 2-related factor 2
NRH	dihydronicotinamide riboside
p	probability value
PARP1	poly-(ADP-ribose) polymerase 1
PBS	phosphate buffered saline
PI	propidium iodide

PPP	pentose phosphate pathway
R123	rhodamine 123
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	room temperature
s	second(s)
SD	standard deviation
SDS	sodium dodecyl sulfate
siRNA	small interfering RNSA
SN	supernatant
SSA	sulfosalicylic acid
t	time
V	volts
v/v	volume per volume
w/v	weight per volume
WST1	water soluble tetrazolium salt 1

1. Introduction

1.1 Brain cells

1.1.1 Neural cells: Functions and interconnections

The brain is made up of an assortment of different cell types that interact with and support each other to allow for incredible performance in cognition and intelligence. The simple transfer of basic electrochemical signals allows for complex outputs like recognition, dreaming or love. Therefore, it is not surprising that a lot of research is focused on this fascinating organ, from overall structural analysis to single molecule interactions, to gain further information and to understand the numerous brain and nervous system located or associated diseases (Sweatt 2016, Verkhratsky and Nedergaard 2016, Verkhratsky and Nedergaard 2018).

In mammals, the central nervous system (CNS) is formed by the spinal cord and the brain, including the retina and the olfactory bulb. The cells within are called neural cells which are further divided into neurons and neuroglia, i.e. the glial cells of the brain (Verkhratsky and Nedergaard 2018). The major neuroglia are microglia, oligodendrocytes, ependymal cells and astrocytes (figure 1.1) (Allen and Barres 2009, Del Bigio 2010, Fields *et al.* 2014).

Neurons are responsible for electrochemical signal transduction by depolarization or hyperpolarization of their membranes. Neuronal signals are entering at the synapses of the dendrites of a neuron and are transmitted by the axon and terminal synapses to the next neurons. This process is essentially fastened by myelination of neuronal processes by oligodendrocytes (Nave 2010). Between two consecutive neurons a tripartite synapse can be formed by the synapse of the presynaptic neuron, the synapse of the postsynaptic neuron and astrocytes (Perea *et al.* 2009). In between the tripartite synapse the synaptic cleft is located, into which neurotransmitters are released by the presynaptic neuron that can bind to receptors of the membrane of the postsynaptic neuron, potentially relaying the initial signal. Astrocytes are known to influence this processing of signals by taking up neurotransmitters and thus clearing the synaptic cleft and even releasing neurotransmitters themselves (Fields *et al.* 2014, Verkhratsky and Nedergaard 2018).

While neurons excel in and are responsible for signal transduction, neuroglia essentially shape the brain environment to favor and ensure neuronal function, as well as modulate it directly (Fields *et al.* 2014). Oligodendrocytes form the isolating myelin sheaths around neuronal axons by wrapping layers of extended cell membrane around them and forming the famous Nodes of Ranvier (Nave 2010). This significantly reduces energy consumption for restoration of ion gradients for neurons, as action potentials and ion currents are restricted to only 0.5 % of the membrane surface as compared to non-myelinated axons, and it increases the conduction velocity up to 100-fold, only first enabling high-end cognition (Nave 2010). Microglia are the immune cells of the CNS and migrate in between other brain cells, constantly monitoring the status of the adjacent cells with their processes (Salter and Stevens 2017). They are not only responsible for acute effects like inflammation and the clearance of cell debris, they also are important in neurogenesis and neuroplasticity, as well as associated with long-term memory and learning (Salter and Stevens 2017). For both types of neuroglia, oligodendrocytes and especially microglia, it holds true that they have been largely neglected in the past but in recent years have gotten way more attention, sparking potent insights and new ideas about their functions in and effects on the brain (Nave 2010, Fields *et al.* 2014, Salter and Stevens 2017).

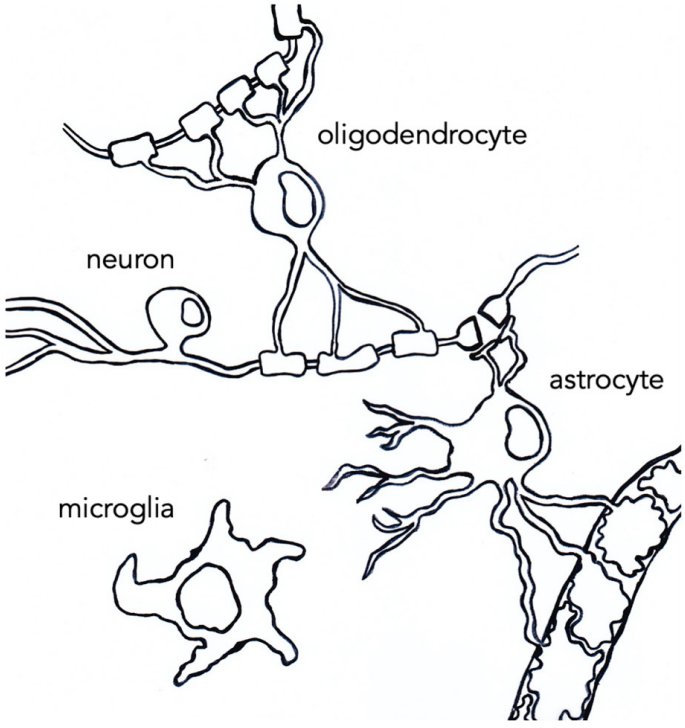

Figure 1.1: Schematic drawing of brain cells and selected interactions.

Besides, ependymal cells form a ciliated epithelium on the brain ventricles. By moving their clustered apical cilia, they constantly move the cerebrospinal fluid, what has been postulated to facilitate diffusion (Del Bigio 2010).

As astrocytes are of specific importance for the presented thesis, this glia cell type will be described in more detail in the following.

1.1.2 Astrocytes

The term astrocyte is derived from the Greek expressions astron (“star”) and kytos (“hollow vessel”; later: “cell”) and literally translates as star-like cell (Verkhratsky and Nedergaard 2018), which actually refers to the very characteristic morphology of only some subtypes of astrocytes (Matyash and Kettenmann 2010). Astrocytes are an essential neural cell type of the brain and originate from the same universal neural progenitor as neurons and oligodendrocytes (Kriegstein and Alvarez-Buylla 2009). They are responsible for an extreme variety of homeostatic processes in the central and peripheral nervous system, are closely involved in neuronal networks and the antioxidative defense of the brain (Lange *et al.* 2012, Dringen *et al.* 2015, Verkhratsky *et al.* 2015, Verkhratsky and Nedergaard 2018).

Astrocytes are a very heterogenous and versatile type of cells and a universal marker does not exist yet, neither in genetic expression nor in morphology (Matyash and Kettenmann 2010, Verkhratsky and Nedergaard 2018). Naturally, therefore ordering of astrocytes into subclasses is under debate, up to the point of philosophical contemplation of the parameters for astrocyte definition (Kimelberg 2004). Nevertheless, as most astrocytes express the intermediate filament glial fibrillary acidic protein (GFAP), and they are the only cells in the CNS expressing it, GFAP is in general accepted as a suitable marker (Verkhratsky and Nedergaard 2018).

Functionally, astrocyte importance has developed from nerve glue passively filling up space not occupied by neurons (Verkhratsky *et al.* 2015), to the fundamental homeostatic cells of the brain, meaning nothing less as astrocytes being involved or responsible in tremendous amount of processes with essential supporting functions and even direct modulation of neuronal activity (Lange *et al.* 2012, Verkhratsky *et al.* 2015).

Astrocytes are engaged in forming the blood-brain barrier. They cover the blood vessels that supply the brain almost completely with their endfeet and control import and export of substances into the brain, which is supposed to keep harmful substances

from entering this susceptible part of the body (Parpura *et al.* 2012, Verkhratsky and Nedergaard 2018). Also, because of the contact to neurons and blood vessels, astrocytes are a central part of the neurovascular unit, that is controlling blood flow in the brain according to neuronal activity and need, by adjusting the diameter of blood vessels and thereby directing oxygen and blood glucose flow (Attwell *et al.* 2010, Muoio *et al.* 2014).

In brain signal cascading, astrocytes are integrated intimately. Different subclasses of astrocytes are connected to distinct parts of neurons and one astrocyte can engulf several synapses with its processes (Allen and Barres 2009). Astrocyte membranes near synapses are densely crowded by neuroreceptors and ion channels, enabling them to take up neurotransmitters and directly communicate with neurons to couple astrocytic internal status to neuronal activity and influence down-stream processing (Verkhratsky and Nedergaard 2016). Exemplary, the turnover of the neurotransmitter glutamate and the prevention of its accumulation in the synaptic cleft is a fundamental function of astrocytes in protecting neurons (Bylicky *et al.* 2018, Verkhratsky and Nedergaard 2018). Perisynaptic astrocytes first clear glutamate from the synaptic cleft and inactivate it to glutamine by glutamine synthetase, to then shuttle glutamine back to neurons to allow for a recycling loop that is saving neurons from complete re-synthesis of glutamate and protects them from excitotoxicity (Bylicky *et al.* 2018, Mahmoud *et al.* 2019).

In brain energy metabolism, astrocytes provide energy for neurons in the form of lactate. Therefore, astrocytes efficiently import glucose by the glucose transporter 1 (GLUT1) and after phosphorylation shuttle it into glucose-dependent pathways (Patching 2017). While glycogenesis can be used to store excess glucose in the form of glycogen (Bélanger *et al.* 2011, Bak *et al.* 2018), lactate produced in glycolysis can be exported from astrocytes by monocarboxylate transporter (MCT) 1 or 4 (Perez-Escuredo *et al.* 2016). Neurons can take up extracellular lactate using MCT2 (Perez-Escuredo *et al.* 2016) and fuel mitochondrial respiration for the production of ATP (Tang *et al.* 2014, Magistretti and Allaman 2018). In the brain, astrocytes are the cells to store most of the glycogen, underlining their supporting role for other brain cells (Bélanger *et al.* 2011, Bak *et al.* 2018).

Astrocytes are also described to protect neurons from mitochondrial dysfunction, by importing damaged mitochondria that then undergo mitophagy in the astrocytes and not in the neuron (Davis *et al.* 2014). Conversely, astrocytes were shown to export healthy mitochondria for neurons *in vivo* to incorporate, most likely in a calcium-dependent manner i.e. coupled to neuronal activity (Hayakawa *et al.* 2016). This

mechanism is thought to work by the formation of tunnelling nanotubes in the membranes of neurons and astrocytes, while the formation of nanotubes in astrocytes is guided by neuronal activity (Bylicky *et al.* 2018).

The antioxidative defence system of astrocytes is a central function of this brain cell type as they also protect neighbouring cells against oxidative stress (Dringen *et al.* 2015). Therefore, this elaborated capacity will be explained in more detail in the later following chapter 1.3.2.

To further increase the knowledge about astrocytes, primary cultures of these cells can be used. In such cultures, astrocytes are enriched over other brain cell types as confirmed by the immunostaining against the astrocyte marker GFAP and contain only low numbers of contaminating cells, mostly microglia and oligodendrocytes (Tulpule *et al.* 2014). This allows studies of this glia cell type in the near absence of non-target cells (Lange *et al.* 2012) and essentially removes the modulating effects of neighbouring cells of the physiological brain to allow the gathering of information about the astrocyte cell type alone (Tulpule *et al.* 2014) and creates a test system for the potential effects of therapeutic drugs on the brain (Arend *et al.* 2013, Westhaus *et al.* 2015).

1.2 Oxidative stress and quinone redox cycling

Living cells fundamentally rely on the principle of homeostasis, i.e. virtually all cellular processes have to be in balance with its respective counterparts. Over as well as under balancing is associated with changes in normally functional equilibria and can lead to dysregulation and disfunction (Modell *et al.* 2015), ultimately followed by cell-death and disease of the whole organism. That is why sophisticated mechanisms on all levels of the cell are in place, to detect deviations from a desirable steady state and subsequently counteract unphysiological changes (Modell *et al.* 2015).

The balance between oxidative and reductive processes is one crucial factor in cell homeostasis, and the burden the cell has to deal with to keep the oxidative processes in balance is called oxidative stress (Sies *et al.* 2017). The responsible chemical species vary immensely in their reactivity and in their original nature: They range from well described reactive oxygen species (ROS) and reactive nitrogen species, to reactive sulfur species, reactive carbonyl species or reactive selenium species (Sies *et al.* 2017). Notably, oxidative stress is normal to a specific extent, i.e. physiological, and not negative as such (Pomatto and Davies 2018). Specifically, certain reactive by-products of metabolism cannot be prevented completely and only be detoxified after their occurrence, e.g. the production of short and long-lived ROS in mitochondrial respiration (Mattson and Liu 2002, Adam-Vizi 2005, Venditti *et al.* 2013) or the production of hydrogen peroxide during fatty acid degradation (Foerster *et al.* 1981, Zhang *et al.* 2019). Also, radicals fulfil important signalling functions in sensing oxidation-reduction states (Reczek and Chandel 2015, Sobotta *et al.* 2015).

Notably, oxidants can also result from the reduction of other appropriate compounds, especially of quinone nature (Sies *et al.* 2017). Quinones have a fully conjugated dione structure and can vary greatly in the attached side groups (Nic *et al.* 2005). The two attached oxygen atoms of the central benzol ring each can accept or release one-electron. In presence of a suitable oxidant, one-electron reduction of a quinone results in the formation of a semi-quinone radical. This can (auto)-oxidize and reduce oxygen or other electron accepting compounds and thereby regenerates the parent quinone and releases a labile superoxide anion (Sies *et al.* 2017). Two-electron reduction of a quinone results in the formation of a generally considered more stable hydroquinone, which can principally auto-oxidize in a two-step reaction via the semiquinone-radical to the parent quinone, releasing two electrons (Klotz *et al.* 2014). Potentially, quinones can undergo infinite cycles of reduction and oxidation steps (Sies *et al.* 2017).

1.2.1 Menadione

Menadione is a quinone compound, chemically termed 2-methyl-1,4-naphtoquinone (Klotz *et al.* 2014). As a quinone, menadione can be reduced to menadione semiquinone radical or menadione hydroquinone (menadiol), depending on the number of electrons transferred, one or two, respectively (figure 1.2). Reduced forms of menadione can also be oxidized back to the respective parent quinone form, releasing one electron per step. Oxidation of menadione semiquinone radical and menadiol happens spontaneously in presence of molecular oxygen, leading to the formation of superoxide and other thereof derived ROS (Klotz *et al.* 2014), while menadione can also be enzymatically reduced (Rooseboom *et al.* 2004). One-electron transfer to menadione producing menadione-semiquinone is catalyzed by cytochrome P450 reductase (Bayol-Denizot *et al.* 2000, Rooseboom *et al.* 2004) or mitochondrial ubiquinone reductase (Floreani and Carpenedo 1992). Two-electron transfer to

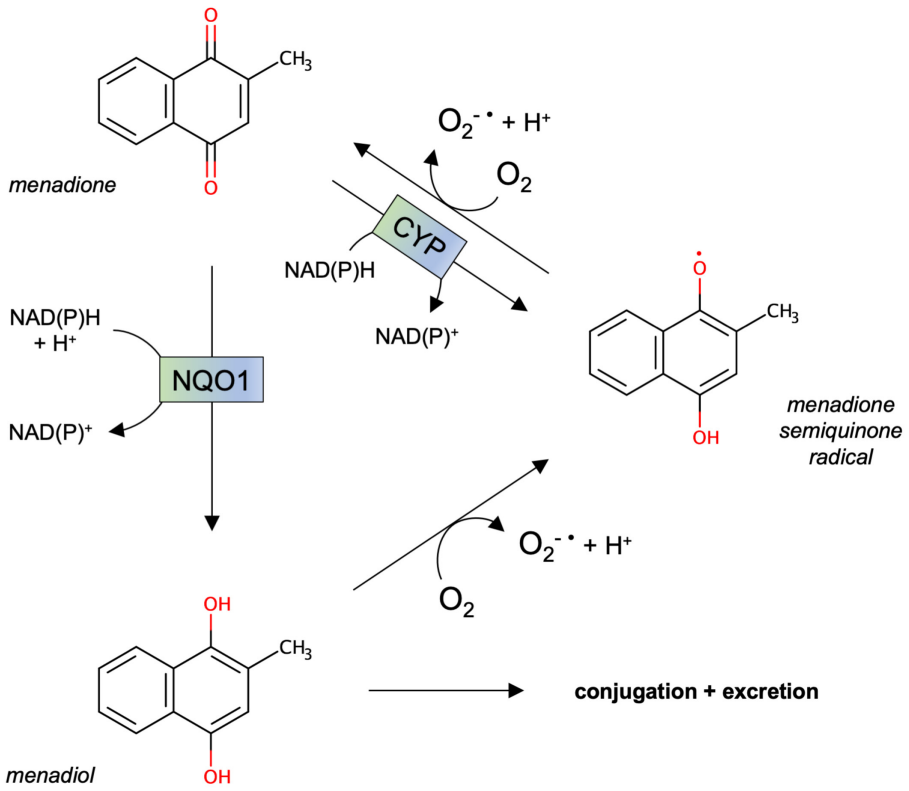

Figure 1.2: Enzymatic reduction of menadione. Menadione can be reduced by a one-electron reduction by cytochrome P450 (CYP) to its semiquinone-radical, which can in turn auto-oxidize back to menadione by releasing superoxide. By a two-electron reduction of menadione by NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) this immediate semiquinone-formation is prevented by generating menadiol (menadione-hydroquinone). Menadiol can also be conjugated to water soluble molecules to facilitate excretion from the cell.

menadione producing menadiol is catalyzed by carbonyl reductase or cytosolic NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) (Rooseboom *et al.* 2004).

Formation of menadiol is in general believed to be beneficial for cells, as menadiol is chemically more stable than menadione semiquinone (Klotz *et al.* 2014). Also, hydroquinone formation is a prerequisite to allow phase II detoxifying enzymes glutathione-*S*-transferases and UDP-glucuronosyl-transferases to couple water soluble moieties to the hydroxyl groups of the hydroquinone and to facilitate excretion (Zhang *et al.* 2013, Klotz *et al.* 2014). Simultaneously, the conjugate of menadione and glutathione, 3-glutathionyl-2-methyl-1,4-naphtoquinone, has been described to show increased auto-oxidation rates in comparison to the unsubstituted menadione hydroquinone and to be also a substrate of NQO1 (Buffinton *et al.* 1989).

Menadione is also termed vitamin K3, as it is a derivative of the lipophilic vitamins K1 (phylloquinone) and K2 (menaquinone), missing the variable length isoprenoid chains (Gong *et al.* 2008). Phylloquinone and menaquinone fulfil important functions in the blood clotting cascade, in bone metabolism and in vascular biology. The hydroquinone form of vitamin K is needed to enzymatically γ-carboxylate glutamic acid residues on blood clotting factors like pro-thrombin and to functionalize the mineral binding protein osteocalcin in bone. Therefore, vitamin K is activated to vitamin K hydroquinone which is used as a substrate of vitamin K-dependent γ-glutamyl carboxylase for carboxylation of glutamic acid residues to functionalize the protein (Stafford 2005, Gong *et al.* 2008). Neither vitamin K can be stored in the body but they are recycled and reused (Gong *et al.* 2008).

Menadione displays antitumor activity in a variety of tumors (Jamison *et al.* 2001, Rooseboom *et al.* 2004) and is investigated as an anti-cancer drug (Ren *et al.* 2019). The anti-tumor activity is believed to be the consequence of menadione-induced redox cycling and superoxide production in cells (Jamison *et al.* 2001, Ren *et al.* 2019).

The characteristic of menadione as a redox cycler also lead to the application of menadione in tetrazolium salt-based assays. Tetrazolium salts are widely used in biochemical applications, as they form brightly coloured formazans upon reduction, that are easy to detect and to quantify due to different spectrophotometric properties to the parental tetrazolium salt (Berridge et al. 2005). For instance, in combination with the well-known mono-tetrazolium salt MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide), menadione has been used to quantify activity of NQO1, using it as the substrate for NQO1 and menadiol to reduce MTT to its coloured and quantifiable MTT formazan (Prochaska and Santamaria 1988). Also, menadione is used in

combination with the water-soluble tetrazolium salt WST1 (5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium salt), to determine metabolic activity of neural cells (figure 1.3) (Stapelfeldt *et al.* 2017, Ehrke *et al.* 2020). Herein, menadione is used as electron cycler in low concentrations to transfer electrons from cellular reduction equivalents to the membrane impermeable WST1 in the incubation medium, allowing for the extracellular formation of a coloured WST1 formazan product (Stapelfeldt *et al.* 2017). It is believed, that NQO1 is the primarily responsible enzyme for cellular menadione reduction in this viability assay, as addition of the NQO1 inhibitor dicoumarol prevents formation of WST1 formazan (Ehrke *et al.* 2020).

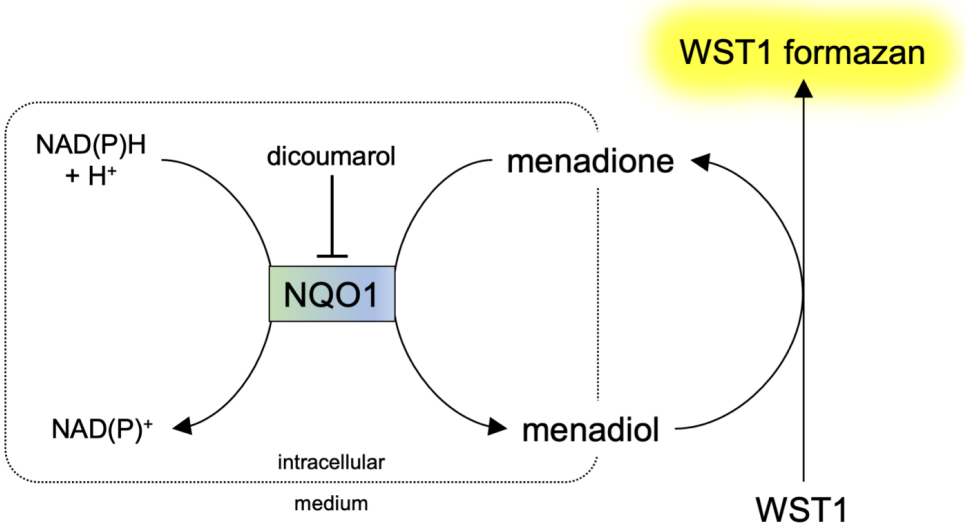

Figure 1.3: Principle of the WST1 reduction assay. Extracellular water soluble tetrazolium salt 1 (WST1) is reduced from intracellular sources of NAD(P)H to its easily detectable WST1 formazan. As WST1 cannot enter the cell, the electron cycling naphthoquinone menadione is used, which is intracellulary reduced by NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) and shuttles electrons over the cell membrane to WST1. The assay is efficiently inhibited by the NQO1 inhibitor dicoumarol.

1.2.2 Beta-lapachone

Beta-lapachone is a naphthoquinone (figure 1.4) which is isolated from trees belonging to the families of bignoniaceae and verbanaceae (Bermejo *et al.* 2017) and analogues are abbreviated ARQ 501 (Bey *et al.* 2007) and ARQ 761 (Beg *et al.* 2017) in clinical use. Like menadione, beta-lapachone can take part in redox cycling and can be reduced to either beta-lapachone semiquinone, by a one-electron reduction, or beta-lapachone hydroquinone, by a two-electron reduction of cellular enzymes as described above. However, beta-lapachone hydroquinone, i.e. beta-lapachol, is not chemically stable and readily auto-oxidizes back to beta-lapachone leading to superoxide formation in the presence of oxygen (Silvers *et al.* 2017). Also, beta-lapachone is a substrate of NQO1 (Bey *et al.* 2013, Silvers *et al.* 2017). In this combination, NQO1-mediated reduction does not lead to detoxification of the substrate, but is actually catalysing the activation of beta-lapachone, leading to increased levels of ROS within cells and ultimately cell death (Pink *et al.* 2000, Chakrabarti *et al.* 2015, Beg *et al.* 2017, Silvers *et al.* 2017), highlighting the two-faced role of NQO1 in detoxifying systems (Ross and Siegel 2017).

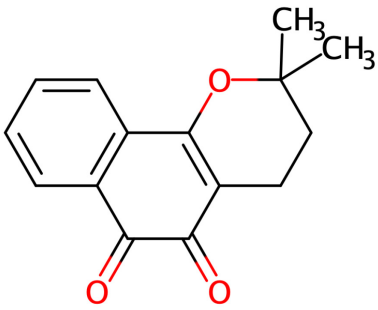

Figure 1.4: Chemical structure of beta-lapachone

This toxic bioactivation is the reason why beta-lapachone is associated with many favourable results, showing induction of toxicity in a large variety of tumour cells (Pink *et al.* 2000, Chakrabarti *et al.* 2015, Huang *et al.* 2016, Beg *et al.* 2017, Silvers *et al.* 2017) and has been used in clinical trials on patients (Bey *et al.* 2007, Beg *et al.* 2017). The NQO1-dependent ROS formation after activation of beta-lapachone causes DNA strand breaks, which reaching severe levels will hyperactivate the DNA-repair enzyme poly-(ADP-ribose) polymerase 1 (PARP1), responsible for the stabilisation and repair of DNA strand breaks (Bey *et al.* 2007, Huang *et al.* 2016). Hyperactivation of PARP1 is followed by large consumption of NAD^+ and severe losses in cellular ATP, eventually leading to programmed cell death (Bey *et al.* 2007).

Due to the high expression of NQO1 in cancerous cells (Belinsky and Jaiswal 1993, Glorieux *et al.* 2016) this is believed to be the primary cause for the anti-tumour activity of beta-lapachone (Kung *et al.* 2014, Beg *et al.* 2017, Bermejo *et al.* 2017).

1.3 Antioxidative defense

The antioxidative defense systems that prevent cellular homeostasis from tipping over under oxidative stress fundamentally rely on the activity of a variety of enzymes that are generally classified as drug metabolizing enzymes (DMEs) (Zhang *et al.* 2013). Based on the sequential order of function DMEs are further divided into phase I, phase II and phase III detoxifying enzymes (Nakata *et al.* 2006). In short, phase I detoxifying enzymes oxidize drugs and xenobiotics, phase II detoxifying enzymes conjugate substances to products of phase I reactions to increase their hydrophilicity and facilitate excretion, while phase III detoxifying enzymes execute the removal of the conjugates from the cell (Nakata *et al.* 2006, Zhang *et al.* 2013). In general, the regulation of expression and degradation of the involved DMEs is complex and diverse. Therefore, only the main regulatory pathway of phase II genes will be explained here, as this includes the regulation of pivotal enzymes involved in glutathione metabolism (Dringen *et al.* 2015, Vriend and Reiter 2015) and the quinone reductase NQO1 (Dinkova-Kostova and Talalay 2010).

The Nrf2/Keap1/ARE-pathway is the main regulator of phase II detoxifying enzymes (Zhang *et al.* 2013), and regulates expression of enzymes directly involved in the detoxification of electrophiles like glutathione-*S*-transferases, glutathione reductase (Vriend and Reiter 2015) or NQO1 (Dinkova-Kostova and Talalay 2010) and of enzymes which generate the essential reduction equivalents required for antioxidative defense like glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the oxidative part of the pentose phosphate pathway (PPP) or malic enzyme (Kensler *et al.* 2007).

Under unstressed conditions the cytosolic kelch-like ECH-associated protein 1 (Keap1) can associate with an ubiquitin ligase (Cul3-Rbx1) and bind to the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2, figure 1.5) (Vriend and Reiter 2015). This prevents Nrf2 from migrating to the nucleus and leads to its ubiquitination by the Keap1-Cul3-Rbx1-complex and subsequently degradation of Nrf2 by the proteasome (Vomhof-Dekrey and Picklo 2012). Under oxidative stress conditions, critical cysteine residues of Keap1 are reduced and it loses the ability to bind Nrf2 (Kensler *et al.* 2007). As a consequence, Nrf2 is not ubiquitinated, can accumulate in the cytoplasm and migrates to the nucleus, where it activates the DNA promoter antioxidative response element (ARE) (Vriend and Reiter 2015).

The central reduction equivalent required to fuel antioxidative defense is NADPH that is provided by the PPP in dependence of the availability of glucose (Dringen *et al.* 2007). In astrocytes, the redox pair NADPH/$NADP^+$ is mostly in the reduced state of NADPH to provide electrons for reductive biosynthesis and is furthermore tightly connected with the redox pair GSH/GSSG, which is directly responsible for antioxidative defense in astrocytes (Dringen *et al.* 2007, Dringen *et al.* 2015). Under unstressed conditions, most of the available glucose is shuttled into glycolysis to fuel catabolic metabolism. However, high cellular demands in NADPH due to oxidative stress trigger a redox switch which re-directs glucose into the oxidative parts of the PPP and sustain antioxidative defense by e.g. allowing the regeneration of GSH from GSSG (Brandes *et al.* 2009, Mullarky and Cantley 2015).

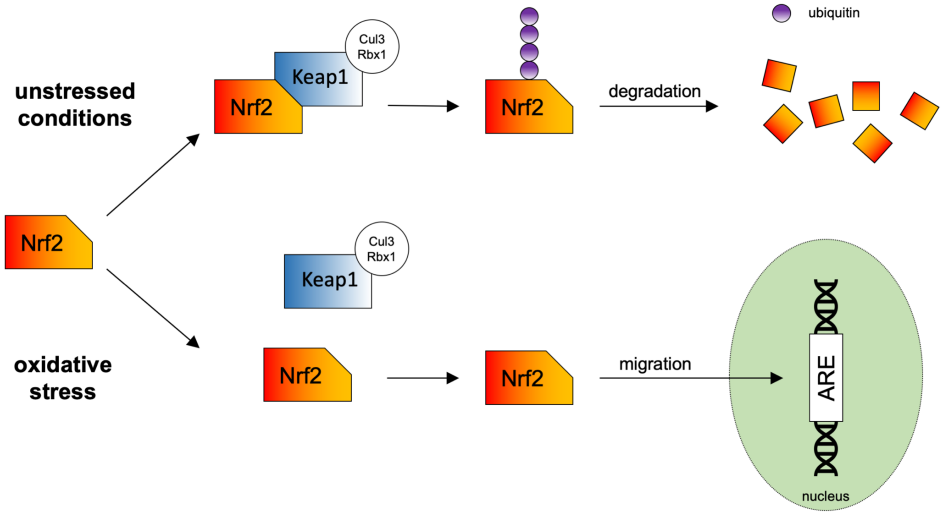

Figure 1.5: Regulation of Nrf2 migration to the nucleus. Under unstressed conditions the transciption factor Nrf2 is bound in the cytosol by Keap1 and the associated ubiquitin ligae Cul3-Rbx1. This prevents Nrf2 migration to the nucleus and leads to proteasomal degradation of Nrf2 after ubiquitination by the Keap1-Cul3-Rbx1-complex. Under oxidative stress, conformational changes prevent Keap1 from binding to Nrf2, allow its accumulation in the cytosol and migration to the nucleus. Here Nrf2 facilitates genetic expression of proteins under the control of the antioxidative response element (ARE).

1.3.1 NAD(P)H: quinone acceptor oxidoreductase 1

The NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1, EC: 1.6.99.2) is an enzyme integrated into the antioxidative defense of cells (Dinkova-Kostova and Talalay 2010, Ross and Siegel 2017, Pey *et al.* 2019) and catalyzes the obligatory two-electron reduction of a variety of substrates including quinones, nitroaromatics or azo dyes (Ernster *et al.* 1962, Lind *et al.* 1990, Ross and Siegel 2017). NQO1 has also been named DT-diaphorase, vitamin K reductase or menadione reductase over the years (Vasiliou *et al.* 2006). It can use both reduced nicotinamide nucleotides, either NADH or NADPH, with equal efficiency as energy substrate (Ernster *et al.* 1962, Ernster *et al.* 1972), which is very unusual for an oxidoreductase (Lind *et al.* 1990, Dinkova-Kostova and Talalay 2010). Expression of NQO1 is found in many animals including rats and humans (Dragan *et al.* 2006, Vasiliou *et al.* 2006) and is regulated by the Keap1/Nrf2/ARE pathway (Dinkova-Kostova and Talalay 2010), which is a major regulator for the expression of proteins connected to cellular antioxidative defense (Kensler *et al.* 2007). Expression of NQO1 is inducible by a variety of compounds including quinones, diphenols, Michael-reaction acceptors, organosulfur compounds, hydroperoxides, trivalent arsenicals, heavy metals and carotenoids (Dinkova-Kostova *et al.* 2004). Also, NQO1 expression is high in a range of tumorous cells (Belinsky and Jaiswal 1993, Glorieux *et al.* 2016), making the enzyme an interesting target for potential cancer treatment (Kung *et al.* 2014, Beg *et al.* 2017). This high activity of NQO1 in some tumor cells is believed to be the consequences of the rapid metabolism associated with a higher load of free radicals and a therefore higher expression of NQO1 (Pey *et al.* 2019).

NQO1 is a homodimeric enzyme with one catalytic center per monomer located at the interface in between them (Li *et al.* 1995, Asher *et al.* 2006). Every monomer contains one non-covalently bound flavin adenine dinucleotide (FAD) as a cofactor. Binding of reduced pyridine nucleotide to the enzymatic pocket starts the catalytic cycle by electron transfer from NAD(P)H to FAD (Hosoda *et al.* 1974). The oxidized pyridine nucleotide afterwards is excluded from the enzyme and leaves a reduced $FADH_2$ in the catalytic pocket. Then, the second substrate, e.g. a quinone, can bind to the same and now empty pocket and is only released from the active side as hydroquinone after complete oxidation of $FADH_2$ to FAD (Hosoda *et al.* 1974, Hollander and Ernster 1975). This is a so-called *ping-pong bi-bi* enzymatic mechanism (Preusch *et al.* 1991), i.e. the second substrate only enters the active site of the enzyme after the first product has already left the enzymatic pocket (Bisswanger 2000). NQO1 is efficiently inhibited by the anticoagulant drug dicoumarol (figure 1.6) in low micromolar concentrations and K_{ic} values in the low nanomolar range (Hosoda *et al.* 1974, Ehrke *et al.* 2020), through

competition with the oxidized pyridine nucleotide substrate (Hollander and Ernster 1975, Chen *et al.* 1999). Structural evidence suggests that dicoumarol binds to the active side of the enzyme and partially overlaps the FAD cofactor (Asher *et al.* 2006), preventing the initial electron transfer from NAD(P)H.

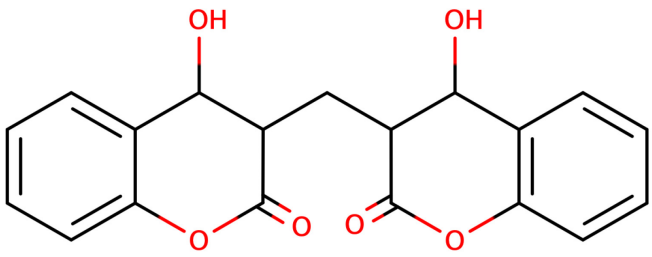

Figure 1.6: Chemical structure of the NQO1 inhibitor dicoumarol.

Cellular localization of NQO1 was found to be mainly cytosolic (Ernster *et al.* 1972, Edwards *et al.* 1980, Siegel *et al.* 2012a), also in primary astrocyte cultures (Ehrke *et al.* 2020). However, as many of the described substrates of NQO1 exhibit lipophilic character or are found in membranes (Lind *et al.* 1990), close association of NQO1 with membranes is likely.

NQO1 is very efficient in reducing various quinones to their respective hydroquinone (Hosoda *et al.* 1974, Lind *et al.* 1990) and thereby prevents the formation of semiquinone radicals, that are inevitably associated with redox cycling and cellular oxidative stress (Ross *et al.* 1993, Rooseboom *et al.* 2004). Because of this, NQO1 activity is reputed to be beneficial for cells in lowering the burden of semiquinone-induced oxidative stress and subsequent drawbacks (Dinkova-Kostova and Talalay 2010, Ross and Siegel 2017). However, the actual consequences of the enzymatic catalysis of NQO1 do depend to a great extent on the chemical stability of the produced hydroquinone, i.e. NQO1 activity can also be responsible for the bioreductive activation of a quinone compound, essentially amplifying its possibly adverse consequences (Ross and Siegel 2017). This is for example the case in the bioactivation of beta-lapachone by NQO1, leading to an unstable beta-lapachone hydroquinone, that is auto-oxidizing back in two steps in presence of molecular oxygen to the parent quinone, leading to superoxide formation and DNA damage (Huang *et al.* 2016, Silvers *et al.* 2017). Then again, hydroquinone formation is necessary and precedes the clearance of the same from cells by conjugation with glutathione or UDP-glucose (Klotz *et al.* 2014). This is making NQO1 activity fundamentally two-sided or in other

words versatile, and the consequences of enzymatic activity exceptionally dependent on the reduced product and the context.

Beneficial quinone reduction by NQO1 is known for physiological substrates ubiquinone (coenzyme Q, CoQ) (Beyer *et al.* 1996) and α-tocopherol quinone, the latter resulting from radical activation of vitamin E (Siegel *et al.* 1997). Both are important components of the plasma membrane redox system, responsible for defense against oxidative stress (Hyun *et al.* 2006). NQO1 was found to protect mitochondrial membranes from damage by free radicals and lipid peroxidation by ensuring reduced states of CoQ_9 and CoQ_{10} (Beyer *et al.* 1996). Notably, short chain CoQ_1 and CoQ_2 were better substrates of NQO1 than long chain CoQ_9 and CoQ_{10} (Beyer *et al.* 1996). Activation of α-tocopherol quinone by NQO1 is generating the potent antioxidant α-tocopherol hydroquinone, which can restore antioxidant activity in a biological system (Siegel *et al.* 1997). It was speculated that the high affinity of NQO1 towards xenobiotics, including quinone derivatives, might only be a secondary function of the enzyme and not its primary purpose (Beyer *et al.* 1996).

The NQO gene family also includes another flavoenzyme that is part of the same cellular response to oxidative stress as NQO1 (Bianchet *et al.* 2004), termed NHR: quinone acceptor oxidoreductase 2 (NQO2) (Long and Jaiswal 2000). NQO2 is 43 amino acid residues shorter at its C-terminus than NQO1 (Bianchet *et al.* 2004), which encompasses a fragment of the pyridine nucleotide binding site as compared to NQO1 (Vasiliou *et al.* 2006). This explains why NQO2 can indeed also utilize NAD(P)H, albeit less efficient than NQO1, and has been shown to prefer dihydronicotinamide riboside (NRH) as cofactor (Wu *et al.* 1997, Bianchet *et al.* 2004). Similar to NQO1, NQO2 also catalyzes two-electron reductions of a variety of substrates including quinones (Wu *et al.* 1997).

1.3.2 Antioxidative functions of astrocytes and glutathione metabolism

The brain is an energy demanding organ and roughly 20% of inhaled oxygen is consumed in the brain (Bolaños 2016). Oxygen consumption is however unavoidably linked to the generation of reactive oxygen species (ROS), that are prone to damage nucleic acids and lipids or lead to disfunction and degradation of proteins and enzymes (Sies *et al.* 2017, Bylicky *et al.* 2018). Astrocytes contain a sophisticated antioxidative defence system that allows them to protect also neighbouring cells, including neurons, from oxidative stress and thereby essentially help establish whole brain homeostasis and protect neuronal function (Dringen *et al.* 2015).

Central in this astrocytic defence system is the low-molecular substance glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine, figure 1.7) that is present in millimolar concentration in astrocytes (Bylicky *et al.* 2018) and in astrocyte cultures of around 8 mM (Dringen and Hamprecht 1998, Dringen 2000).

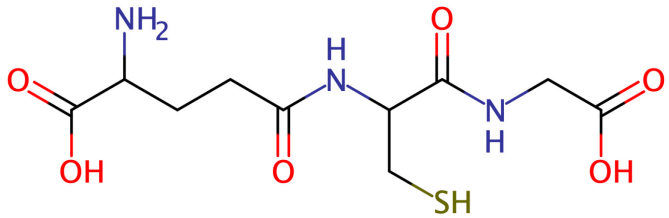

Figure 1.7: Chemical structure of GSH (γ-L-glutamyl-L-cysteinylglycine).

The tripeptide GSH is synthetized from the three amino acids glutamate, cysteine and glycine in two distinct ATP-dependent enzyme catalysed reactions. First glutamate cysteine ligase combines glutamate and cysteine to the dipeptide γ-glutamylcysteine, by an isopeptide bond between the terminal carboxyl group of glutamate and the amine group of cysteine. Second glutathione synthetase adds the amino acid glycine to generate GSH (Lu 2013). The supply of glutamate, cysteine and glycine for GSH synthesis is backed by extracellular uptake of the amino acids or cellular generation from various precursor molecules, including other amino acids and peptides (Lu 2009, Lu 2013, Dringen *et al.* 2015). Glutamate can be taken up into astrocytes by excitatory amino acid transporters (EAAT) 1 and 2 from the extracellular space (Had-Aissouni 2012), while glycine is efficiently imported by the glycine transporter GlyT1 (Szoke *et al.* 2006). Cysteine is easiest imported into astrocytes as cystine, the disulfide form of cysteine, by the cystine glutamate antiporter and then reduced in the cell to cysteine (Kranich *et al.* 1996, Kranich *et al.* 1998).

Antioxidative reactivity of GSH towards radicals and peroxides is divided into distinct functions (figure 1.8). On the one hand, GSH can directly react with oxidants and concomitantly reduce them to less harmful species without enzymatic involvement. On the other hand, GSH serves as substrate for glutathione peroxidases, enzymes responsible for detoxification of organic peroxides or hydrogen peroxide (Dringen *et al.* 2015, Dwivedi *et al.* 2020). Independent from the mechanism, GSH-dependent reduction of oxidants results in the formation of glutathione disulfide (GSSG). As the redox pair GSH/GSSG is the central antioxidant of most aerobic organisms and represents the main redox pair of the cellular thiol pool (Deponte 2013, Montero *et al.* 2013), cellular accumulation of GSSG would lead to an undesirable shift in the cellular thiol redox potential (Sies *et al.* 2017). Under unstressed conditions the ratio of GSH to GSSG in astrocytes is very high and GSSG is nearly not detectable (Dringen and Hamprecht 1997, Bishop *et al.* 2007). However, under oxidative stress conditions, cellular concentrations of GSSG increase, disturbing the cellular thiol redox balance. Therefore, to decrease cellular GSSG and allow the glutathione-system to efficiently work against oxidants, GSSG can be exported from astrocytes by the ATP-dependent multidrug resistance protein 1 (Mrp1) (Hirrlinger *et al.* 2001, Minich *et al.* 2006) or it can be recycled and reduced to GSH by NADPH-dependent

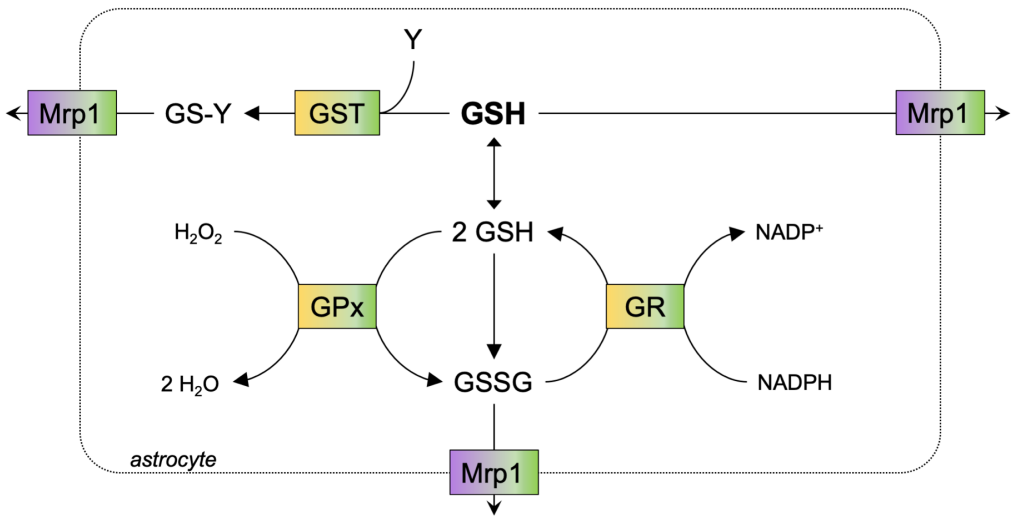

Figure 1.8: Main functions and metabolism of GSH in astrocytes. GSH is the central antioxidant in astrocytes and is constantly exported from the cytosol to the extracellular space by multidrug resistance protein 1 (Mrp1). It can be non-enzymatically oxidized to detoxify electrophiles or can be used by glutathione peroxidase for the reduction of hydrogen peroxide. Both reactions lead to the formation of glutathione disulfide (GSSG), that is efficiently exported by Mrp1 or can be recycled to GSH by glutathione reductase at the expense of NADPH. Additionally, GSH can be conjugated to various xenobiotics (Y) by glutathione-*S*-transferases forming a glutathione-xenobiotic-conjugate (GS-Y) that is also exported by Mrp1.

glutathione reductase to ultimately re-establish cellular high ratio of GSH to GSSG (Dringen *et al.* 2015) that is necessary for glutathione-dependent enzymatic function.

As a third option GSH can be conjugated to xenobiotics by glutathione-*S*-transferases (Board and Menon 2013). This conjugation of the thiol group of GSH to the electrophile may lead to a better solubility of xenobiotics, depending on the respective properties, and facilitates their active elimination from the cell by Mrp1 that is exporting glutathione conjugates, too. In general, glutathione-conjugates are considered less toxic than the parent molecule (Dallas *et al.* 2006, Keppler 2011).

GSH is exported constantly from astrocyte cultures by Mrp1 (Hirrlinger *et al.* 2002c, Minich *et al.* 2006). Here, GSH can be cleaved by the extracellular enzyme γ-glutamyl transpeptidase, yielding precursor molecules that can be taken up by neurons and used for neuronal GSH synthesis. In relation, astrocytes constantly synthesize GSH, as long as enzymatic substrates are available and cellular GSH concentrations are below a certain threshold (Dringen *et al.* 2015).

Taken together, the overall specific content of GSH in astrocytes is determined by various interdependent factors. Lowering of the cellular GSH content is the consequence of an active export of GSH by Mrp1, the oxidation of GSH to GSSG due to oxidative stress and/or the conjugation of GSH to xenobiotics. Raises of the cellular GSH content are due to new synthesis of GSH, inhibition of GSH export and due to the recycling of GSH from GSSG by glutathione reductase.

1.4 Aim of the thesis

The quinone-compounds menadione and beta-lapachone are both substrates of the cellular enzyme NQO1 that will enzymatically reduce these substrates to their respective hydroquinone form (Ernster *et al.* 1962, Pink *et al.* 2000, Rooseboom *et al.* 2004, Silvers *et al.* 2017). Both, menadione and beta-lapachone, are of clinical relevance given their anti-tumor potential (Jamison *et al.* 2001, Rooseboom *et al.* 2004, Chakrabarti *et al.* 2015, Beg *et al.* 2017, Bermejo *et al.* 2017, Ren *et al.* 2019) and are therefore an interesting target for studies on brain cells that might be exposed to these quinones upon treatment of patients. In the present thesis, astrocyte primary cultures are used as a model system of astrocyte brain cells (Lange *et al.* 2012, Tulpule *et al.* 2014) and will be studied for the consequences of a treatment with the quinones menadione and beta-lapachone.

First, given the central role of NQO1 in the reduction of quinones, astrocyte primary cultures will be tested for their expression of NQO1. This will be conducted by the detection of NQO1 enzymatic activity in lysates of astrocytes and will be confirmed using the NQO1 inhibitor dicoumarol, that efficiently blocks NQO1 activity (Hollander and Ernster 1975, Pey *et al.* 2019). Additionally, presence of NQO1 will be proven by Western blotting of astrocyte lysates and immunofluorescent staining of intact astrocyte cultures for NQO1.

Second, the vitality of astrocytes after exposure to menadione or beta-lapachone will be analyzed. The extracellular accumulation of lactate released from cultured astrocytes will be used as a marker for metabolic activity, while the release of cellular LDH from astrocytes will be used as a marker for the loss in membrane integrity (Tulpule *et al.* 2014).

Third, the quinones menadione and beta-lapachone will be separately tested for their potential to induce ROS formation in astrocytes and for their consequences on the glutathione metabolism. As both menadione (Abe and Saito 1996, Kesari *et al.* 2020) and beta-lapachone (Park *et al.* 2016) have been described to induce ROS in cells the oxidation of the important cellular antioxidant GSH (Dwivedi *et al.* 2020) on application of the compounds to astrocytes cultures will be quantified. The occurrence of ROS will be investigated using ROS-mediated formation of fluorescent dyes while the consequences on the cellular GSH metabolism will be evaluated by measuring the cellular and extracellular contents of GSx and GSSG upon treatment of astrocytes with menadione or beta-lapachone. For consequences on the glutathione metabolism as well as on the vitality of astrocytes upon exposure to menadione and beta-lapachone,

the NQO1-inhibitor dicoumarol will be used as a confirmation of an involvement of NQO1 (Hollander and Ernster 1975, Pey *et al.* 2019).

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2. Experimental results

2.1 Investigation of expression of NAD(P)H: quinone acceptor oxidoreductase 1 in astrocyte primary cultures from rat brain

Investigation of expression of NAD(P)H: quinone acceptor oxidoreductase 1 in astrocyte primary cultures from rat brain

Johann Steinmeier

Contributions of Johann Steinmeier:

- All data and figures shown
- Writing of the chapter

Abstract

NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) is an enzyme that is interconnected to the antioxidative defense of cells. NQO1 prevents the one-electron reduction of quinone compounds to its respective semiquinone radical that subsequently causes the formation of reactive oxygen species (ROS). By obligatory two-electron reduction, NQO1 produces the hydroquinone of the quinone-substrate that can be conjugated and more easily excreted from the cell. NQO1 has been postulated to be the responsible enzyme for the menadione-dependent reduction of water-soluble tetrazolium salt 1 in astrocyte-rich primary cultures. To test for the presence of NQO1, the activity of NQO1 in lysates from astrocyte cultures was determined. Also, Western blotting of astrocyte lysates for NQO1 was performed and immunochemical staining for NQO1 in intact astrocyte cultures. It was demonstrated that astrocyte hypotonic lysates contain a high specific enzymatic activity of NQO1 determined to be 342 ±12 nmol/(min*mg) that could almost completely be blocked by the NQO1-inhibitor dicoumarol. Also, antibody detection of NQO1 after Western blotting of astrocyte lysates revealed definite signals, confirming the presence of NQO1. Additionally, immunochemical detection of NQO1 in intact astrocyte cultures showed a broadly distributed signal with dot-like characteristics and more intensive signals around condensed chromosomes. In summary, the presented results demonstrate the presence of NQO1 in rat astrocyte primary cultures.

Introduction

NQO1 is an oxidoreductase, which is associated with antioxidative defense (Dinkova-Kostova and Talalay 2010) and is expressed in a wide range of tissue in eukaryotic organisms (Vasiliou *et al.* 2006). Supposedly, NQO1 activity is beneficial for cells because its enzymatic mechanism prevents the formation of radical products by obligatory two-electron reduction onto its substrates (Dinkova-Kostova and Talalay 2010). One of the various substrates of this enzyme are quinones (Ernster *et al.* 1960, Ross and Siegel 2017), which are prone to take part in redox cycling and the formation of superoxide that is subsequently followed by different reactive oxygen species (Sies *et al.* 2017). The one-electron reduction of quinones by e.g. cytochrome P450 reductases lead to the formation of a semiquinone radical (Rooseboom *et al.* 2004). NQO1 is preventing the production of a semiquinone-radical that is directly associated with superoxide production and redox cycling (Ross *et al.* 1993, Rooseboom *et al.* 2004) by only releasing the parental quinone after the transfer of two-electrons and the formation of a hydroquinone has taken place (Hosoda *et al.* 1974, Hollander and Ernster 1975). However, the actual consequences of NQO1 catalysis do depend to a great extent on the chemical properties and the stability of the produced hydroquinone, i.e. NQO1 catalysis can also lead to the activation of a quinone facilitating adverse consequences (Ross and Siegel 2017). NQO1 is effectively inhibited by dicoumarol, a competitive inhibitor of NQO1 with respect to NAD(P)H, with K_{ic}-values in the nanomolar range (Hollander and Ernster 1975, Chen *et al.* 1999, Ehrke *et al.* 2020).

Astrocytes are the most abundant cells in the central nervous system (Argente-Arizon *et al.* 2017) with many essential functions (Verkhratsky and Nedergaard 2018). For one, astrocytes contain a sophisticated antioxidative defense system that allows them to protect neighboring cells against xenobiotics that have crossed the blood-brain barrier and help in the defense against oxidative stress (Dringen *et al.* 2015, Bolaños 2016). This includes the expression of antioxidative enzymes that are under the control of the ARE promoter and the Nrf2-pathway, like NQO1 (Dinkova-Kostova and Talalay 2010), which is lower expressed in neurons than in astrocytes (Ahlgren-Beckendorf *et al.* 1999, Bell *et al.* 2015).

NQO1 activity is found elevated in a range of tumor cells (Begleiter *et al.* 2004, Kung *et al.* 2014, Huang *et al.* 2016, Beg *et al.* 2017, Silvers *et al.* 2017) and is also found in rat astrocytes (Dragan *et al.* 2006, Ehrke *et al.* 2020). Recent work in our group relies on NQO1 being responsible in astrocyte-rich primary cultures as the main responsible enzyme in the metabolic WST1 assay and in the toxic bioactivation of beta-

lapachone (Ehrke *et al.* 2020, Steinmeier *et al.* 2020). Therefore, presence of NQO1 in rat primary cultures was investigated by determining NQO1 enzymatic activity and by immunochemical methods.

Material and Methods

Material

Dulbecco's modified Eagle's medium (DMEM; containing 25 mM glucose) and penicillin/streptomycin solution was purchased from Gibco (Darmstadt, Germany), fetal calf serum from Biochrom (Berlin, Germany) and bovine serum albumin (BSA) from AppliChem (Darmstadt, Germany). Reagents and film for Western blotting were from GE Healthcare (Munich, Germany). The primary monoclonal anti-actin antibody (mouse) was from Dianova (Hamburg, Germany), the primary polyclonal anti-NQO1 antibody (rabbit) was from Sigma, the secondary antibodies against mouse and rabbit were from Dianova and the Cy2-conjugated goat anti-rabbit antibody was purchased from Jackson ImmunoResearch (West Grove, Pennsylvania, USA). The Page ruler restrained protein ladder 10-170 kDa was from ThermoFisher Scientific (Bremen, Germany). All other chemicals of the highest purity available were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany) or Riedel-de Haën (Seelze, Germany). Sterile 5 cm cell culture dishes were from Sarstedt (Nümbrecht, Germany).

Methods

Astrocyte-rich primary cultures

Astrocyte-rich primary cultures were prepared according to a described protocol (Hamprecht and Loffler 1985, Tulpule *et al.* 2014). Newborn Wistar rats were decapitated within24 h after birth and the full brains were pressed through nets to remove blood vessels and singularize cells. For seeding, 5 mL of cell suspension containing 600 000 cells/mL cell culture medium (90 % DMEM, 10 % FCS, 1 mM pyruvate, 18 U/mL penicillin G and 18 µg/mL streptomycin sulfate) were transferred into 5 cm dishes. For immunostaining, cells were seeded in a 24 well plate with 1 mL cell culture medium per well containing 300 000 cells/mL on top of sterile glass coverslips The medium was renewed every 7th day and one day prior to every experiment. The cultures were used at an age between 14 and 28 days. For maintenance of the cultures 5 cm dishes were incubated at 37 °C and a humidified atmosphere in an incubator (Sanyo, Osaka, Japan).

The procedure according to this protocol results in astrocyte cell cultures with minimal contaminations of oligodendrocytes, microglia and neurons but 95 % of cells being positive for the astrocyte-specific marker protein GFAP (Tulpule *et al.* 2014).

Cell lysate preparation by hypotonic lysis

Cell lysates from astrocyte-rich primary cultures cultured on 5 cm dishes were obtained by hypotonic lysis and fractionation of the resulting suspension by centrifugation according to a published method (Ehrke *et al.* 2020). Hypotonic lysis relies on the swelling of the cell by osmotic pressure and the subsequent disruption of the cell membrane (Baumgarten and Feher 2012).

Here, astrocytes cultured in 5 cm dishes were placed on an ice-filled tray and were washed once with 2 mL ice-cold (4°C) phosphate buffered saline (PBS; 10 mM potassium-phosphate buffer, pH 7.4) and once very fast with 2 mL ice-cold (4°C) hypotonic lysis buffer (HEPES: 20 mM, pH 7.3 adjusted with 1 M NaOH). After the removal of the buffer, the cells were lysed for 30 min on ice in 1 mL hypotonic lysis buffer. The full lysate was then obtained by scraping off the cells from the dish and mixing of the suspension with a pipette tip. Then, the full lysate was centrifuged for 5 min at 4 °C in a tabletop centrifuge (Minispin, Eppendorf, Germany) at 12 100 g. Afterwards, the lysate supernatant (SN) was collected and the pellet was resuspended in the exact same volume.

Determination of lysate NQO1 activity

Determination of lysate NQO1 activity was performed on the basis of the NQO1-dependent reduction of water-soluble tetrazolium salt 1 (WST1) in presence of the electron cycler menadione to its colored WST1 formazan as described previously (Stapelfeldt *et al.* 2017, Ehrke *et al.* 2020).

Briefly, 10 µL of lysate SN after hypotonic lysis of astrocytes was mixed to final concentrations with 5 mM NADH, 70 µM WST1 and 100 µM menadione. Then, the enzymatic activity of NQO1 was determined from the initial linear increase in WST1 formazan absorbance at 405 nm ($\varepsilon_{405\,nm}$: 24.135 mM^{-1} cm^{-1}) (Stapelfeldt *et al.* 2017) measured by a microtiter plate reader (Tecan, Grödig, Austria). As a control the NQO1 inhibitor dicoumarol (Hollander and Ernster 1975) was used in a final concentration of 1 µM.

The significance of differences between calculated NQO1 activities in absence and presence of dicoumarol was determined with a paired *t*-test ($^{###}p<0.001$), as the experiments were conducted on three independently prepared astrocyte cultures.

Western Blot Analysis

Western blots to test for the presence of NQO1 in astrocyte-rich primary cultures were performed as previously described (Hohnholt *et al.* 2011, Bulcke and Dringen 2015) with only few alterations. The samples from hypotonic lysis of astrocytes from 5 cm dishes were first heated for 10 min at 95 °C and then mixed with sample buffer (0.312 M Tris/HCl, pH 6.8, 10 % (w/v) SDS, 50 % (v/v) glycerine, 0.05 % (v/v) bromphenol blue) and 20 mM dithiothreitol in water in a ratio of 3:1:1, respectively. The samples were then loaded on polyacrylamide gels (stacking gel 4 %, separation gel 14 %) and electrophoresis was executed for 15 min at 90 V and 1 h at 160 V. Afterwards, blotting was performed onto Amersham Hybond™-C Extra nitrocellulose membrane (GE Healthcare, Munich, Germany) for 1 h at 360 mA. Later the membrane was incubated with polyclonal anti-NQO1 antibody (rabbit, 1:5 000) and monoclonal anti-actin antibody (mouse, 1:20 000), both in blocking buffer (10 mM Tris/HCl, 150 mM NaCl, 5 % (w/v) milk powder) at room temperature (RT) for 2 h. After washing and blocking of unspecific binding sides by addition of 5 % (w/v) milk powder to the washing buffer, the membrane was incubated with peroxidase-conjugated anti-rabbit (1:20 000) and anti-mouse (1:20 000) secondary antibodies at RT for 1 h. After subsequent washing, the blot was developed with the ECL Western blot detection reagents (GE Healthcare) onto an Amersham Hyperfilm ECL X-ray film (GE Healthcare). The protein content of the fractions obtained by hypotonic lysis were measured in accordance with the method by Lowry *et al.* (1951).

Immunostaining of astrocytes

Immunostaining for NQO1 was performed as previously described (Stapelfeldt *et al.* 2017). Therefore, astrocytes on coverslips were washed with ice-cold (4 °C) PBS and fixed with 250 µL 3.5 % (w/v) paraformaldehyde in PBS at RT for 15 min. Then the coverslip was washed three times with 500 µL PBS and permeabilized with 250 µL 0.1 °% (w/v) Triton X-100 in PBS for 15 min. Afterwards, the coverslip was washed again three times with 250 µL PBS and incubated at RT for 1 h with the primary anti-NQO1 antibody (rabbit, 1:200 dilution) in PBS containing 10 %

(v/v) goat serum). Then the cells were washed again three times with 500 µL PBS and incubated with the secondary Cy2-labeled-anti-rabbit antibody (1:200 dilution in PBS containing goat serum) at RT for 1 h in the dark. Next, cell nuclei were stained with DAPI (1 µg/mL in PBS) at RT for 10 min, washed three times with 500 µL PBS and embedded on top of a glass slide with “Mowiol” mounting medium (2.4 g polyvinyl alcohol, 6 g glycerol dissolved in 18 mL 150 mM Tris/HCl buffer pH 8.5) (Stapelfeldt *et al.* 2017). For fluorescence images Eclipse TE2000-U fluorescence microscope with a DS-QiMc camera and imaging software NIS-Elements BR (Nikon, Düsseldorf, Germany) using the following filter settings for Cy2 (excitation at 465-495 nm, emission at 505-515 nm, dichromatic mirror at 505 nm) and for DAPI (excitation at 330-380 nm, emission at 420 nm, dichromatic mirror at 400 nm).

Results

For the confirmation of presence of NQO1 in rat astrocyte primary cultures, astrocyte lysates were analyzed for NQO1 enzymatic activity and by Western blotting, as well as immunocytochemical staining of intact cultures for NQO1.

Determination of NQO1 enzymatic activity in astrocyte cultures revealed a specific activity of 342 ±12 nmol/(min*mg) in the SN (control, figure 2.1). Addition of 1 µM of the NQO1 inhibitor dicoumarol to the lysate SN diminished NQO1 activity to 8 ±4 nmol/(min*mg), which accounts to only 2 % of the initial control activity (figure 2.1).

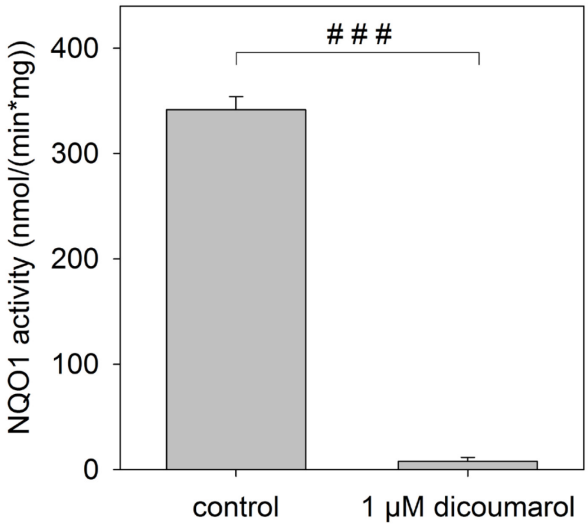

Figure 2.1: NQO1 activity in astrocyte lysate SN and its inhibition by dicoumarol. Astrocyte hypotonic lysates were centrifuged, and the supernatant (SN, control) was tested for NQO1 activity in the absence (control) or presence of 1 µM dicoumarol by measuring the menadione-dependent reduction of the water-soluble tetrazolium salt 1 to its coloured formazan. The protein content of the SN was determined as 889 ± 78 µg/mL. The results were obtained from three independently prepared astrocyte cultures. The significance of differences between the enzyme activities was analysed by a paired *t*-test ($^{###}$<0.001).

For Western blotting, hypotonic lysates from astrocytes were prepared and checked for the presence of NQO1. The full hypotonic lysate (full), the supernatant after hypotonic lysis (SN) and the resuspended pellet (figure 2.2 a) were loaded on a polyacrylamide gel in samples containing 10, 3 and 1 µg protein and stained for actin and NQO1 (figure 2.2 b).

The signals for actin (~ 42 kDa) and NQO1(~ 28 kDa) were detected at the expected molecular masses. The signal intensity for actin in all three fractions tested increased

with the protein amount loaded and was comparable between fractions (figure 2.2 b). Full fraction and SN of hypotonic lysates from astrocytes showed distinct signals for NQO1, and the signal intensity increased with increasing protein content of the samples (figure 2.2 b). For the resuspended pellet only a weak signal for NQO1 was observed for the highest protein amount loaded onto the gel (figure 2.2 b).

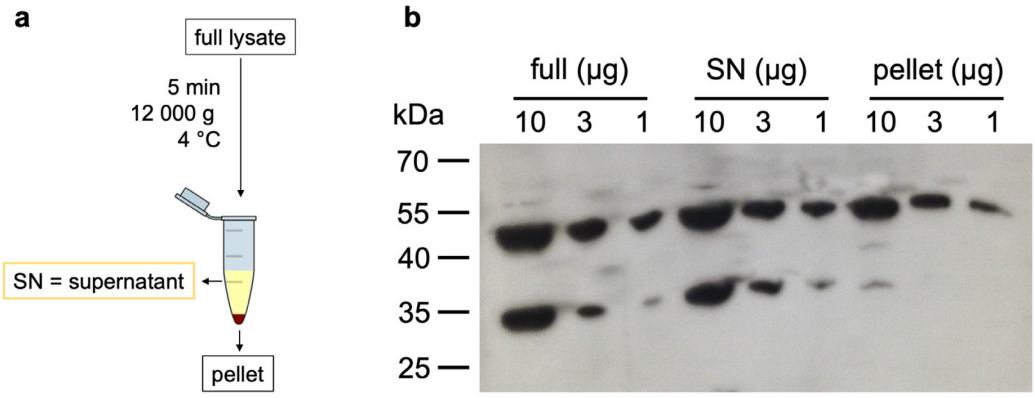

Figure 2.2: Western blot for NQO1 in astrocyte cultures. Astrocytes were cultured in 5 cm dishes and lysed by hypotonic lysis (a). Then a portion of the full lysates was fractioned into supernatant (SN) and pellet after. The full lysate, the SN and the resuspended pellet were then loaded on a polyacrylamide gel for electrophoresis and the gel was blotted onto a nitrocellulose membrane and immunostained for NQO1 (~28 kDa) and actin (~42 kDa) (b). The Western blot shown is a representative example and was reproduced twice on lysates prepared from independent astrocyte cultures.

Immunocytochemical staining of astrocyte cultures revealed a broadly distributed signal for the presence of NQO1 that showed dot-like characteristics to some extent (figure 2.3). The most intense signal was repeatedly found on the cell outside-facing part of condensed chromosomes of mitotic cells as exemplary shown in figure 2.3 (arrow). Controls without the primary antibody against NQO1 or without the secondary antibody Cy2, but with an otherwise identical staining procedure, revealed no notable signals (data not shown).

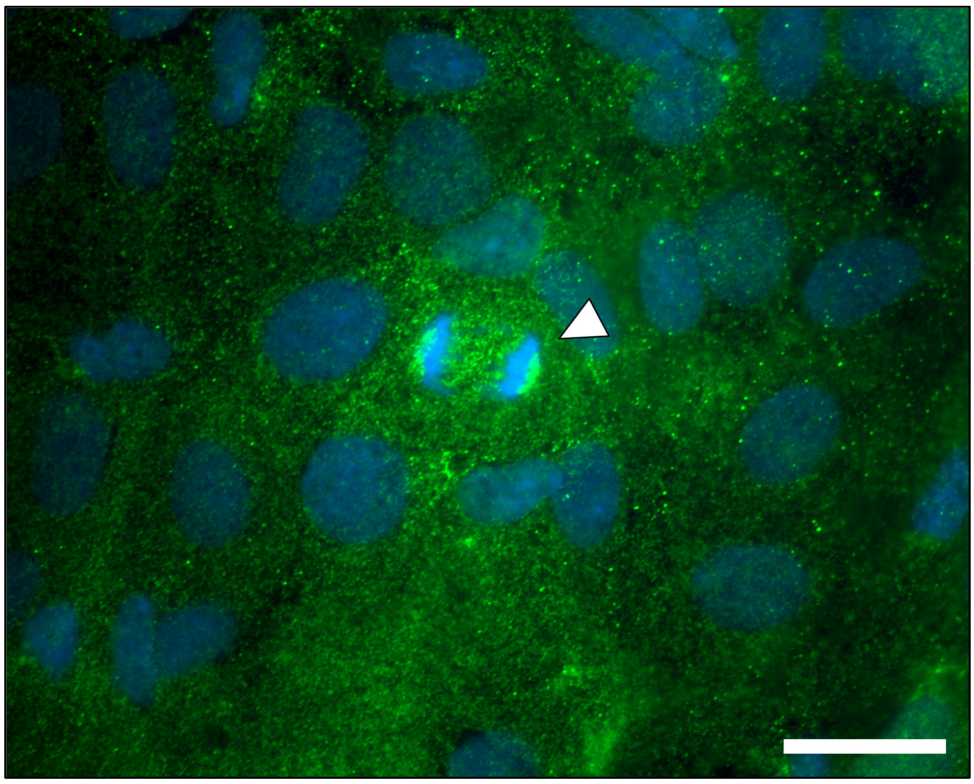

Figure 2.3: Immunocytochemical staining of astrocyte-rich primary cultures for NQO1. Astrocyte cultures were stained for NQO1 (green) and with DAPI (blue) to visualise cell nuclei. Controls without the primary antibody against NQO1 or without the secondary antibody Cy2, but with an otherwise identical staining procedure, revealed no notable signals (data not shown). The scale bar represents 20 µm.

Discussion

The data shown confirm that NQO1 is present in rat astrocyte-rich primary cultures by antibody detection and measurement of enzymatic activity. Importantly, visual confirmation by Western blotting and immunocytochemical staining was achieved, which differs methodically from the so far carried out identification of NQO1 in astrocyte cultures mostly by determination of enzymatic activity and its inhibition by dicoumarol (Stapelfeldt *et al.* 2017, Ehrke *et al.* 2020), a known potent NQO1 inhibitor (Hollander and Ernster 1975, Dinkova-Kostova and Talalay 2010).

Enzymatic activity of NQO1 in astrocytes was proved in accordance with the inhibition by the competitive NQO1 inhibitor dicoumarol (Hollander and Ernster 1975). Dicoumarol competes with the reduced nicotinamide cofactor for binding in the enzymatic pocket of NQO1 with a K_{ic} value in the low nanomolar range (Hosoda *et al.* 1974), in astrocytes determined to be 1.2 nM for NADH and 5.9 nM for NADPH (Ehrke *et al.* 2020). This also excludes NQO2 from being the responsible enzyme for the reduction of menadione used for the activity determination of NQO1, as NQO2 is not noteworthily inhibited by 1 µM dicoumarol (Wu *et al.* 1997). In fact 10 µM dicoumarol inhibit only 25 % of NQO2 activity, and that even in the presence of the NQO2-favoured substrate dihydronicotinamide riboside as co-factor and not the less-favoured NAD(P)H (Wu *et al.* 1997, Long and Jaiswal 2000).

In comparison to literature data, NQO1 activity determined in astrocyte cultures was found to be high, notably also in comparison to the NQO1 activity detected in neurons (table 2.1). In general, it is well known that NQO1 activity is elevated in tumorous cells as compared to normal cells (Belinsky and Jaiswal 1993, Huang *et al.* 2016). The likely reason for this is a higher radical load in rapidly metabolizing cancer cells (Pey *et al.* 2019) and the subsequent upregulation of the Nrf2/Keap1/ARE-pathway for antioxidative protection that is also controlling NQO1 expression (Dinkova-Kostova and Talalay 2010). Neurons, however, are described to have a weak Nrf2-pathway and a low antioxidative capacity in comparison to astrocytes (Ahlgren-Beckendorf *et al.* 1999, Bell *et al.* 2015) and are therefore dependent on astrocytic antioxidative defense (Baxter and Hardingham 2016, Vicente-Gutierrez *et al.* 2020). This is in line with the comparably high NQO1 activity determined in rat astrocyte cultures in this study as compared published data of cultured neurons (Ahlgren-Beckendorf *et al.* 1999, Jia *et al.* 2008).

Table 2.1: Reported NQO1 activities in some cell types.

cell type	NQO1 activity (nmol/(min*mg)	Reference
astrocytes (primary cultures)	~ 340[a]	this chapter
breast tumor (MCF-7)	~ 933[b]	(Glorieux *et al.* 2016)
breast tumor (tissue)	~ 105[b]	(Marin *et al.* 1997)
colon carcinoma (HCT116)	~ 94[d]	(Begleiter *et al.* 2004)
embryonic kidney (HEK239)	~ 10[c]	(Nishiyama *et al.* 2010)
glial cells (primary cultures)	~ 90[b]	(Ahlgren-Beckendorf *et al.* 1999)
lung tumor (tissue)	~ 141[b]	(Marin *et al.* 1997)
neuroblastoma (SH-SY5Y)	~ 9[c]	(Jia *et al.* 2008)
neurons (primary cultures)	~ 6[c]	(Jia *et al.* 2008)
neurons (primary cultures)	~ 35[b]	(Ahlgren-Beckendorf *et al.* 1999)
normal breast (tissue)	~ 54[b]	(Marin *et al.* 1997)
normal lung (tissue)	~ 25[b]	(Marin *et al.* 1997)

Method used for determination of NQO1 activity:
[a] menadione as electron cycler to reduce WST1
[b] menadione as electron cycler to reduce cytochrome c
[c] reduction of 2,6-dichloroindophenol (DCIP)
[d] menadione as electron cycler to reduce MTT

Detection of NQO1 signal after Western blotting allowed to show a protein-dependent increase in the signal of full hypotonic lysates of astrocytes and also the supernatant of hypotonic lysates after centrifugation. The level of NQO1 signal in the corresponding pellet was visibly lower in relation to the protein content loaded, leading to the assumption, that only low amounts of NQO1 are present in the pellet after hypotonic lysis, which should contain mostly water-insoluble cell components like membranes. This fits to the characterization of NQO1 as a mostly cytosolic enzyme (Ernster *et al.* 1972, Edwards *et al.* 1980, Siegel *et al.* 2012a) and also the observation of only minor amounts of NQO1 activity in mitochondrial fractions (Ernster *et al.* 1962) also after digitonin lysis, as compared to the cytosolic fraction from rat astrocyte cultures (Ehrke *et al.* 2020).

Immunocytochemical staining for NQO1 in astrocyte cultures revealed a distributed general signal in the cultured cells, confirming the presence of NQO1 in astrocyte cultures and hinting towards a cytosolic localization. However, the dot-like structure of the immunostaining could also hint towards a vesicular localization of NQO1 in

astrocytes cultures and should be further elucidated. This also includes additional studies to confirm co-localization of NQO1 signal with immunochemical staining for the astrocyte-marker glial acidic fibrillary protein (GFAP) (Tulpule *et al.* 2014) to confirm localization of NQO1 in astrocytes. Intriguingly, immunocytochemical staining signal of NQO1 in astrocyte cultures was more intense in mitotic cells displaying condensed chromosomes, concentrated on the side of the chromosomes facing the exterior of the cells. This is consistent with the finding that NQO1 is associating with the mitotic spindle of cells undergoing division in several human cell lines, including non-transformed astrocytes (Siegel *et al.* 2012a). Furthermore, it was shown recently that NQO1 co-localizes and binds to the mitosis regulator sirtuin SIRT2, an NAD^+-dependent deacetylase primarily found in the cytosol (Kang *et al.* 2018). SIRT2 has responsibilities in chromatin condensation and mitotic exit and is moreover also associating with the mitotic spindle. The role of NQO1 in this complex has been proposed to be the modulation of NADH/NAD^+ ratio and providing NAD^+ in direct vicinity to SIRT2, which needs NAD^+ for guiding mitosis progression (Kang *et al.* 2018).

In summary, the presented results confirm the presence of NQO1 in rat astrocyte primary cultures. This is consistent with the assumption of NQO1 to be the mainly responsible enzyme in the metabolic WST1 assay established on astrocytes (Stapelfeldt *et al.* 2017) as well as the mainly responsible enzyme for the toxic activation of beta-lapachone in astrocytes (Steinmeier *et al.* 2020).

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2.2 Publication 1

Exposure of cultured astrocytes to menadione triggers rapid radical formation, glutathione oxidation and Mrp1-mediated export of glutathione disulfide.

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Neurochemical Research 44: 1167-1181 (2019)

https://doi.org/10.1007/s11064-019-02760-1

Contributions of Johann Steinmeier:

- All data and figures shown
- Preparation of the first manuscript draft

Neurochemical Research
https://doi.org/10.1007/s11064-019-02760-1

ORIGINAL PAPER

Exposure of Cultured Astrocytes to Menadione Triggers Rapid Radical Formation, Glutathione Oxidation and Mrp1-Mediated Export of Glutathione Disulfide

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Received: 13 December 2018 / Revised: 19 February 2019 / Accepted: 20 February 2019

Abstract
Menadione (2-methyl-1,4-naphthoquinone) is a synthetic derivative of vitamin K that allows rapid redox cycling in cells and thereby generates reactive oxygen species (ROS). To test for the consequences of a treatment of brain astrocytes with menadione, we incubated primary astrocyte cultures with this compound. Incubation with menadione in concentrations of up to 30 µM did not affect cell viability. In contrast, exposure of astrocytes to 100 µM menadione caused a time-dependent impairment of cellular metabolism and cell functions as demonstrated by impaired glycolytic lactate production and strong increases in the activity of extracellular lactate dehydrogenase and in the number of propidium iodide-positive cells within 4 h of incubation. In addition, already 5 min after exposure of astrocytes to menadione a concentration-dependent increase in the number of ROS-positive cells as well as a concentration-dependent and transient accumulation of cellular glutathione disulfide (GSSG) were observed. The rapid intracellular GSSG accumulation was followed by an export of GSSG that was prevented in the presence of MK571, an inhibitor of the multidrug resistance protein 1 (Mrp1). Menadione-induced glutathione (GSH) oxidation and ROS formation were found accelerated after glucose-deprivation, while the presence of dicoumarol, an inhibitor of the menadione-reducing enzyme NQO1, did not affect the menadione-dependent GSSG accumulation. Our study demonstrates that menadione rapidly depletes cultured astrocytes of GSH via ROS-induced oxidation to GSSG that is subsequently exported via Mrp1.

Keywords Astrocytes · GSSG · Menadione · Mrp1 · Oxidative stress · Radicals

Introduction

Menadione (2-methyl-1,4-naphthoquinone) is a synthetic derivative of vitamin K [1]. As a quinone, menadione can be reduced in one- and two-electron transfer reactions. The one-electron reduction of menadione generates menadione semiquinone radical, which is prone to take part in cellular redox cycling and in the formation of superoxide which can ultimately cause cell toxicity due to oxidative stress [2]. The two-electron reduction of menadione generates menadione hydroquinone which can be eliminated from cells after phase II enzyme-mediated coupling to glutathione (GSH) or glucuronic acid [2, 3] and subsequent export of the conjugates. The one-electron reduction of menadione to the unstable semiquinone is catalysed by cytochrome P450 monooxygenases, whereas the cytosolic NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) can catalyze the two-electron reduction of menadione [4], thereby preventing the formation of a semiquinone intermediate and being beneficial for cells [5].

GSH is one of the major cellular antioxidants [6–9]. It can directly react with radicals such as superoxide and delivers electrons for the reduction of peroxides by glutathione peroxidases [6]. The product of such reactions is glutathione disulfide (GSSG) which is reduced in cells to GSH in the NADPH-dependent reaction, catalyzed by glutathione reductase [6, 9].

In brain, astrocytes have a key function in the GSH metabolism and in the protection against reactive oxygen

Bremen, Germany

Published online: 26 February 2019

species (ROS) and xenobiotics [6]. Astrocytes contain GSH in millimolar concentrations [7, 10] and the ratio between GSH and GSSG is very high in unstressed cells [11, 12]. Several compounds have been reported to deplete astrocytes of GSH including alkylating substances such as iodoacetamide [13], 3-bromopyruvate [14] or dialkyl-fumarates [15]. Transient depletion of cellular GSH was also found for astrocytes exposed to peroxides [11] or catecholamines [16] due to the rapid oxidation of GSH to GSSG and subsequent export of GSSG. Predominantly responsible for the export of GSH, GSSG and GSH-conjugates from astrocytes is the multidrug resistance protein 1 (Mrp1) [17–22].

In menadione exposed cells, alterations in GSH metabolism have to be considered. The formation of superoxide during menadione redox-cycling can, in addition to directly causing oxidative damage to biomolecules, also weaken the cellular defence against oxidative stress by depleting cellular levels of GSH and NADPH [23]. In addition, during menadione hydroquinone elimination the concentration of the cellular GSH pool can be lowered by conjugate formation [2]. Finally, menadione can directly react with GSH and form a 3-glutathionyl-2-methyl-1,4-naphthoquinone [2, 24], that can also be a substrate of NQO1 and has been reported to even autoxidise faster than the unsubstituted menadione after enzymatic reduction [25].

Menadione has frequently been applied to cultured astrocytes to induce ROS-formation [26–28] and menadione is known for its toxic potential on astrocytes [29–31]. Very recently we have reported that application of 100 µM menadione to cultured astrocytes causes rapid GSH oxidation and GSSG export from viable astrocytes that was prevented by Mrp1 inhibitors [32]. In order to bring this initial observation into a broader context and to substantially extend the current knowledge on the consequences of a menadione exposure of astrocytes, we have investigated now in detail the time- and concentration-dependencies of the toxicity, the ROS formation, the GSSG accumulation and the Mrp1-mediated GSSG export from cultured astrocytes after application of menadione. In addition, we provide experimental evidence showing that ROS formation and cellular GSSG accumulation occurs during glucose deprivation already at lower micromolar concentrations of menadione and that the enzyme NQO1 is unlikely to be involved in the menadione-induced ROS formation and GSSG accumulation in astrocytes.

Materials and Methods

Material

Menadione and dicoumarol were purchased from Sigma (Steinheim, Germany) and MK571 from Biomol (Hamburg, Germany). Powder for the preparation of Dulbecco's Modified Eagle's Medium (DMEM with 25 mM glucose) and penicillin/streptomycin solution were obtained from Gibco (Darmstadt, Germany). Fetal calf serum (FCS) was purchased from Biochrom (Berlin, Germany) and dimethyl sulfoxide (DMSO) from VWR Chemicals (Darmstadt, Germany). The enzymes glutathione reductase, lactate dehydrogenase (LDH) and glutamate pyruvate transaminase were obtained from Roche (Mannheim, Germany). Other chemicals of the highest available purity were from AppliChem (Darmstadt, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Riedel-de Haën (Seelze, Germany) or Sigma (Steinheim, Germany). Sterile 24-well plates for cell culturing and non-sterile 96-well microtiter plates were purchased from Sarstedt (Nümbrecht, Germany).

Astrocyte Cultures

Astrocyte-rich primary cultures were prepared from the total brains of new born Wistar rats and cultured as previously described in detail [33]. The harvested cells were seeded in a density of 300,000 viable cells per well of 24-well plates in 1 mL of culture medium (90% DMEM, 10% FCS, 1 mM pyruvate, 18 U/mL penicillin G and 18 µg/mL streptomycin sulfate) and were cultivated at 37 °C with 10% CO_2 in the humidified atmosphere of a cell incubator (Sanyo, Osaka, Japan). The culture medium was renewed every 7th day and on the day before an experiment was performed. The cultures contain predominantly astrocytes and only minor amounts of oligodendrocytes and microglial cells [33, 34]. Experiments were performed on confluent cultures of an age between 14 and 28 days.

Cell Incubations

The consequences of an exposure of cultured astrocytes with menadione were investigated for incubations of up to 6 h with menadione in concentrations of up to 200 µM. Stock solutions of menadione were prepared in DMSO and diluted in glucose-containing incubation buffer (IB: 20 mM HEPES, 5 mM D-glucose, 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4, pH 7.4) to the final concentrations applied in the cell experiments. Within one type of experiment the final concentration of DMSO was kept constant to 0.05% (Fig. 1, 2, 3, 5, 6, 7, 8) or 0.1% (Fig. 4). For the incubations, astrocytes were washed twice with 1 mL of prewarmed (37 °C) IB and were then incubated for the given incubation periods at 37 °C in a cell incubator without CO_2 supply in 200 µL IB containing menadione and/or other compounds in the concentrations indicated. The incubation was terminated by harvesting the incubation medium and by washing the cells with 1 mL icecold (4 °C)

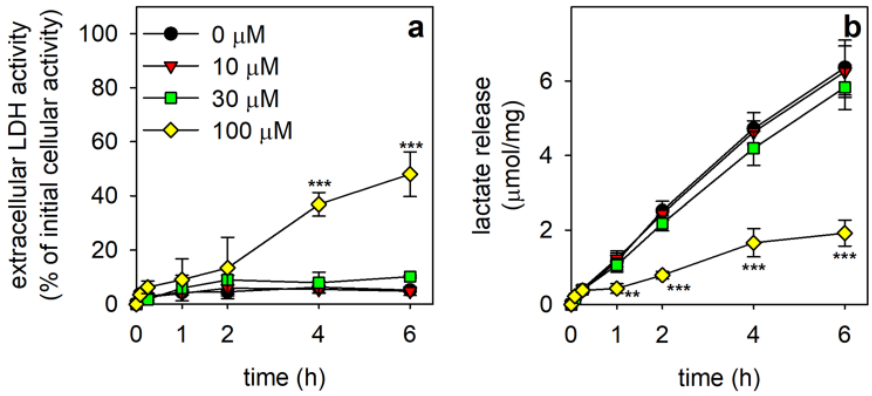


Fig. 1 Effects of menadione on the cell membrane integrity and lactate production. Cultured astrocytes were incubated without or with 10, 30 or 100 µM menadione for up to 6 h and the activity of extracellular LDH (**a**) and the content of lactate released by the cells (**b**) were determined. The initial cellular LDH activity (100%) accounted for 1777 ± 118 nmol/(min × mg) and the protein content of the cultures was 129 ± 3 µg/well. The data shown represent means ± SD of results that were obtained in experiments performed on three independently prepared cultures. The significance of differences compared to the values obtained for the control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (**p < 0.01, ***p < 0.001)

phosphate-buffered saline (PBS: 10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4).

For some experiments the cells were deprived of glucose. For such experiments, the cultures were washed with 1 mL of prewarmed (37 °C) glucose-free IB, then preincubated for 20 min in 200 µL glucose-free or glucose-containing (5 mM) IB and then incubated for the indicated main incubation periods without or with menadione and/or other compounds in glucose-free or glucose-containing IB at 37 °C.

Quantification of Glutathione and Glutathione Disulfide

Total glutathione (GSx = amount of GSH plus twice the amount of GSSG) and GSSG in cell lysates and incubation media were determined by a microtiter-based assay as previously described in detail [33], which is based on the original cycling method published by Tietze [35]. The washed cells were lysed on ice with 200 µL 1% (w/v) sulfosalicylic acid and the lysates were used to determine GSx and GSSG. For quantification of the extracellular contents of GSx and GSSG, 20 µL of a 1:1 mixture of the harvested medium and 1% (w/v) sulfosalicylic acid were used in the cycling assay.

Tests for Cell Viability

The viability of astrocyte cultures after a given treatment was investigated by determining the extracellular activity of LDH in 10 µL medium samples and by propidium iodide staining as describe previously [33]. Extracellular LDH activity is given as percent of the initial cellular LDH activity that was determined for untreated control cells after lysis in 200 µL IB containing 1% (v/v) Triton X100. For propidium iodide staining the treated cultures were washed twice with 1 mL of prewarmed (37 °C) IB and then incubated for 15 min at 37 °C with 500 µL propidium iodide staining solution (5 µM propidium iodide plus 10 µM H33342 in IB). Subsequently, the cells were washed with IB, and fluorescence images were recorded with an Nikon Eclipse TE2000U fluorescent microscope with a DSQiMc camera and the imaging software NIS-Elements BR (Nikon, Düsseldorf, Germany) using the following filter settings for propidium iodide (excitation: 510–560 nm; emission: 590 nm; dichromatic mirror: 575 nm) and for H33342 (excitation: 330–380 nm; emission: 435–485 nm; dichromatic mirror: 400 nm). For all images shown in one multipanel figure, identical microscopic settings and image processing was applied. The fluorescence intensity of the images recorded after PI and H33342 staining was quantified using the software ImageJ and the relative PI fluorescence was calculated without any blank or background correction by normalizing the PI fluorescence to the H33342 fluorescence.

Lactate and Protein Quantification

Glycolytic activity of astrocytes was quantified by determining the extracellular accumulation of lactate in 10 µL samples of the incubation medium by a coupled enzymatic assay system with LDH and glutamate pyruvate transaminase as described previously in detail [33, 36]. The protein content of the cultures was determined according to the Lowry method [37] using bovine serum albumin as standard protein.

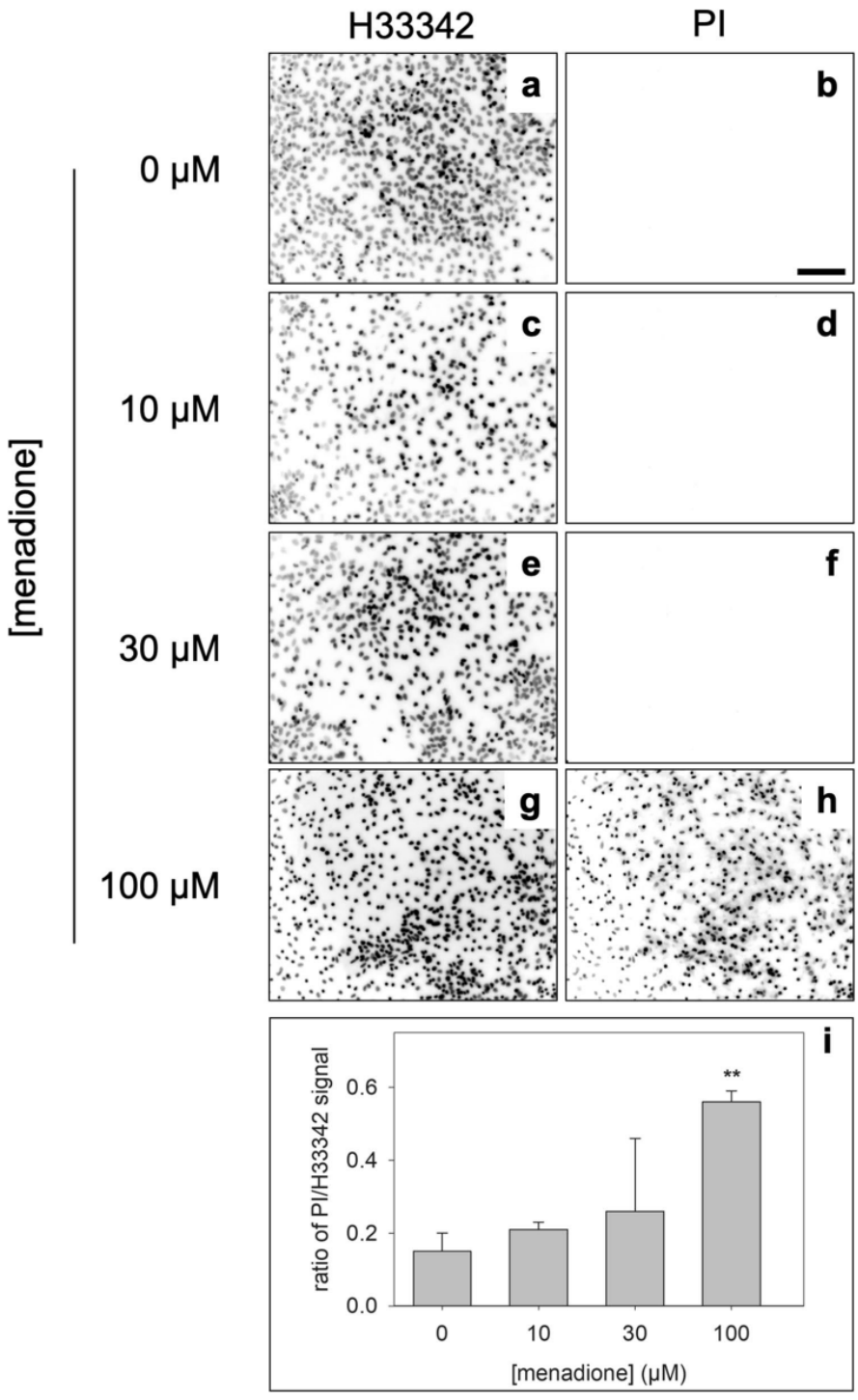


Fig. 2 Propidium iodide staining of astrocytes after menadione treatment. The cultures were incubated for 4 h with menadione in the indicated concentrations and stained with propidium iodide (PI; b, d, f, h) and Hoechst 33342 (H33342; a, c, e, g) to test for membrane permeability and to visualize the total number of cell nuclei, respectively. The scale bar in panel b represents 100 µm and applies to all panels. Panel i shows the relative PI fluorescence (ratio of the PI to H33342 fluorescence intensity signals). The data presented in panel i are means ± SD of results that were obtained in experiments performed on three independently prepared cultures. The significance of differences compared to the values obtained for the control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (**$p < 0.01$)

Test for ROS Formation

Cellular formation of ROS was investigated by visualizing the ROS-dependent oxidation of dihydrorhodamine123 to the fluorescent rhodamine123 by a modification of a published method [38]. The treated cells were washed twice with 1 mL of prewarmed (37 °C) glucose-free IB and then incubated for 30 min at 37 °C with 250 µL dihydrorhodamine123

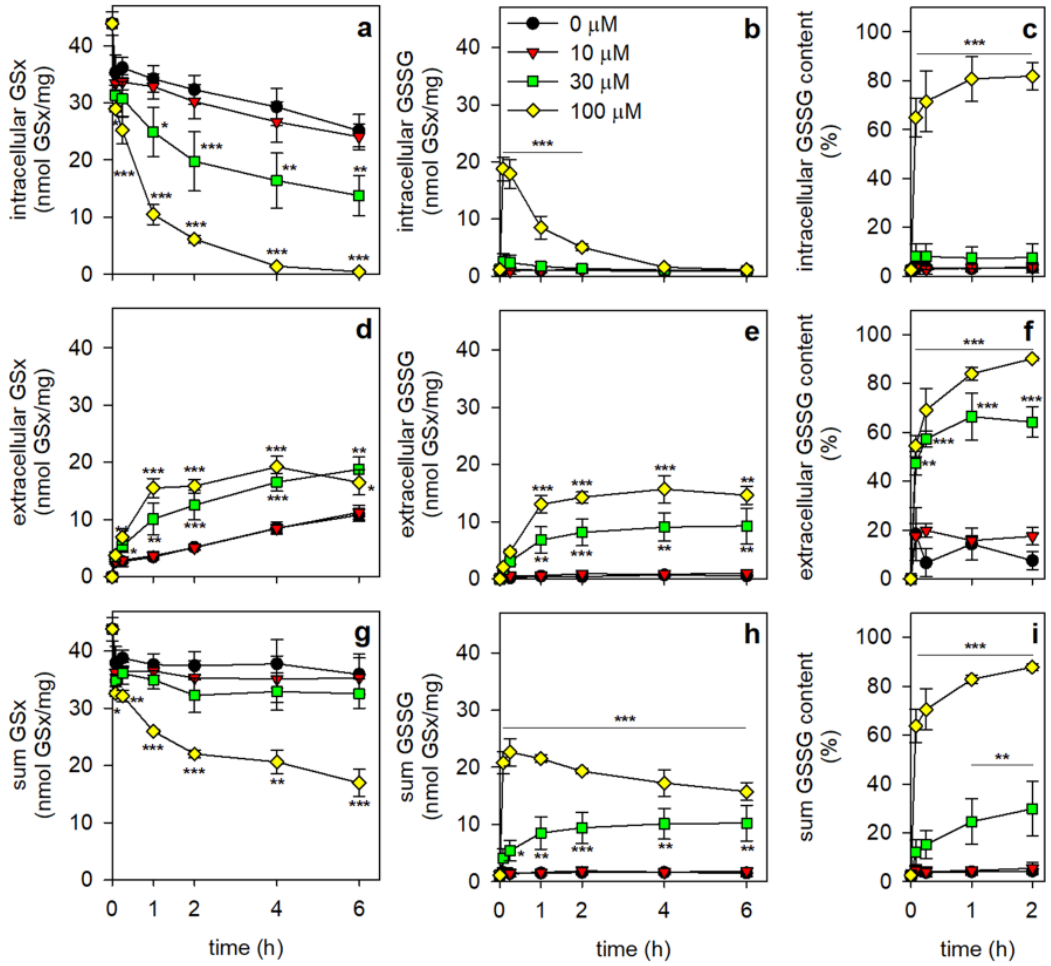


Fig. 3 Consequences of a menadione treatment on the GSx and GSSG content. Cultured astrocytes were incubated without or with 10, 30 or 100 µM menadione for up to 6 h. For the indicated time periods, the intra- and extracellular contents of GSx (**a, d**) and GSSG (**b, e**) were determined. In addition, the sum of intra- and extracellular GSx (**g**) and GSSG (**h**) was calculated. Moreover, the percental contribution of GSSG to the GSx values determined (**c, f, i**) were calculated for the initial 2 h of incubation as no loss in membrane integrity was observed for this time period (see Fig. 1). The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content was 44 ± 2 nmol/mg, the initial cellular GSSG content 1 ± 1 nmol GSx/mg and the initial protein content 129 ± 3 µg/well. The significance of differences compared to the values obtained for the control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

staining solution (5 µg/mL dihydrorhodamine123 plus 10 µM H33342 in glucose-free IB). Finally, the cells were washed twice with 1 mL prewarmed (37 °C) IB and analysed for fluorescence on the Eclipse TE2000U fluorescent microscope using the following filter settings: rhodamine123 (excitation: 465–495 nm; emission: 505–515 nm; dichromatic mirror: 505 nm) and H33342 (excitation: 330–380 nm; emission: 435–485 nm; dichromatic mirror: 400 nm). For all images shown in one multipanel figure, identical microscopic settings and image processing was applied. The fluorescence intensity of the images recorded after staining for rhodamine123 and for H33342 was quantified using the software ImageJ and the relative rhodamine123 fluorescence was calculated without any blank or background correction by normalizing the rhodamine123 fluorescence to the H33342 fluorescence.

Statistical Analysis

All quantitative data shown are means ± standard deviations of values that were obtained in experiments performed on three independently prepared astrocyte cultures.

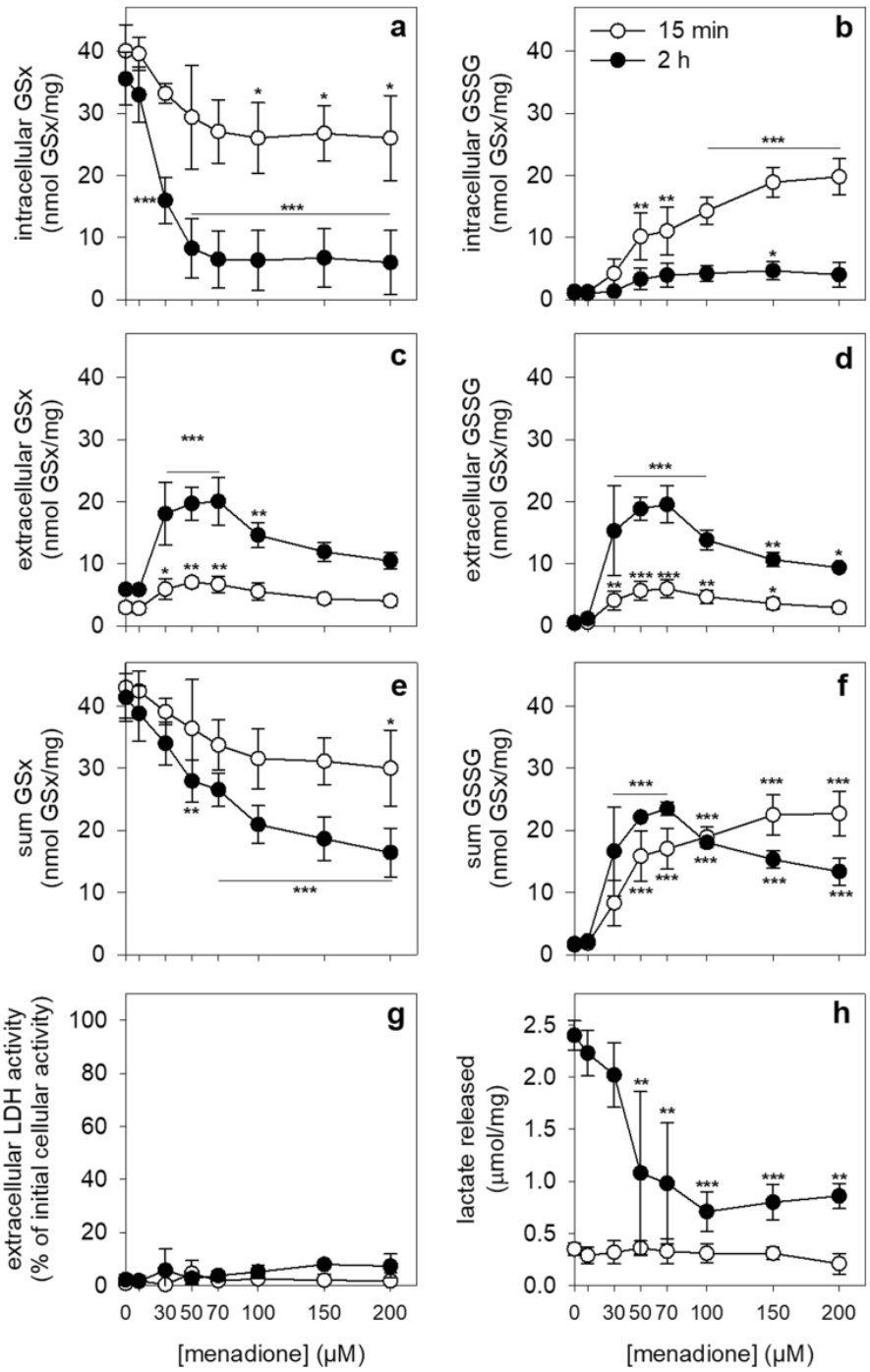


Fig. 4 Concentration-dependent modulation of astrocytic GSH metabolism by menadione. The cultures were incubated without or with the indicated concentrations of menadione for 15 min or 2 h and the intracellular and extracellular contents of GSx (**a, c**) and GSSG (**b, d**), the extracellular LDH activity (**g**) and the extracellular lactate content (**h**) were determined. In addition, the sum of intra- and extracellular GSx (**e**) and GSSG (**f**) were calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 46 ± 5 nmol/mg, the initial cellular GSSG content 1 ± 1 nmol GSx/mg, the initial cellular LDH activity 1622 ± 155 nmol/(min × mg) and the initial protein content 118 ± 25 µg/well. The significance of differences compared to the values obtained for the respective control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Differences between three or more sets of data were tested for significance by analysis of variance (ANOVA) followed by a Bonferroni post hoc test and the statistically significant differences are indicated by asterisks. Significance of differences between two sets of data was analysed by the paired *t* test and the level of significance is indicated by hashes. p values above 0.05 were considered as not significant. The images of stained cells presented in one multipanel figure were obtained in a representative

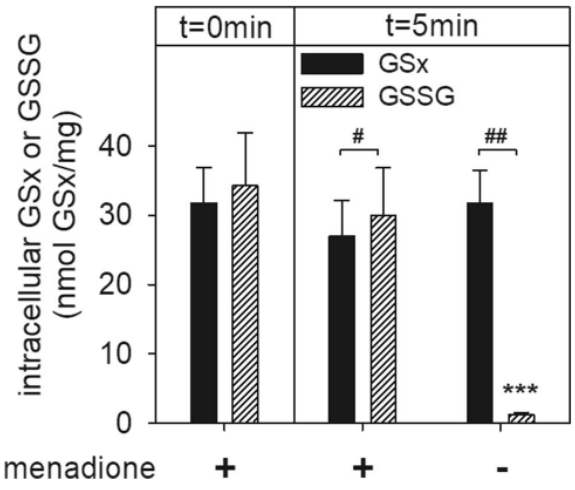


Fig. 5 Reduction of intracellular GSSG after removal of menadione. The cultures were pre-incubated for 5 min with 100 µM menadione (t = 0 min) and subsequently incubated for 5 min without or with 100 µM menadione (t = 5 min) before the cellular contents of GSx and GSSG were determined. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content was 30 ± 3 nmol/mg, the initial cellular GSSG content 0 ± 0 nmol GSx/mg and the initial protein content 99 ± 14 µg/well. The significance of differences compared to the values obtained after the 5 min pre-incubation was analysed by ANOVA and is indicated by asterisks (***$p < 0.001$). Significance of differences between the data obtained for the specific values of GSx and GSSG of one condition was analysed by a paired *t* test and the level of significance is indicated by hashes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

experiment that was reproduced on two independently prepared astrocyte cultures with almost identical outcome.

Results

Menadione-Mediated Toxicity

To test for the potential toxicity of a menadione treatment, cultured primary astrocytes were incubated without or with 10, 30 or 100 µM menadione for up to 6 h and the release of cellular LDH and lactate into the medium was investigated (Fig. 1). Incubations without menadione or with menadione in concentrations of up to 30 µM did not cause any significant increase in extracellular LDH activity (Fig. 1a), in the number of PI-positive cells (Fig. 2b, d, f), in the relative PI fluorescence (Fig. 2i) nor any significant alteration in lactate production (Fig. 1b). In contrast, cells metabolism and membrane integrity were strongly affected during extended incubations of astrocytes with 100 µM menadione, as indicated by the lowered extracellular lactate values found already after 1 h of incubation (Fig. 1b), as well as by the strong increase in extracellular LDH activity, in the number of PI-positive cells and in the relative PI fluorescence of the culture observed after incubation for more than 2 h (Figs. 1a, 2h, i). These data demonstrate that exposure to 100 µM menadione leads to an impairment of astrocytic glucose metabolism and a loss in membrane integrity that are detectable after incubations for 1 h and 4 h, respectively.

Menadione-Induced GSSG Formation and GSSG Export

To test for the potential of menadione to affect the GSH metabolism of astrocytes, the cultures were incubated without or with 10, 30 or 100 µM menadione for up to 6 h. During incubation of astrocytes in the absence of menadione a slow but constant loss in intracellular GSx content was observed (Fig. 3a) that was accompanied by a corresponding increase in extracellular GSx levels (Fig. 3d), resulting in a sum of cellular plus extracellular GSx contents that was almost constant during incubations for up to 6 h and remained almost identical to the initial cellular GSx content of untreated cultures (Fig. 3g). Similar results were observed for astrocytes that had been exposed to 10 µM menadione (Fig. 3). For these conditions, hardly any GSSG was observed in cell lysates and incubation media (Fig. 3b, e, h). In contrast, a concentration- and time-dependent loss in cellular GSx contents and an accelerated appearance of GSx in the medium was observed for cells that had been exposed to 30 µM or 100 µM menadione (Fig. 3a, d). The sum of cellular plus extracellular GSx contents remained almost identical to the values for the control cells (incubation in the absence of menadione) for astrocytes that had been incubated with menadione in concentrations of up 30 µM. In contrast, a significant loss by around 60% of the initial GSx content was observed for cultures that had been incubated with 100 µM menadione for 6 h (Fig. 3g). Incubation of astrocytes with 30 µM and 100 µM menadione caused a mild and strong transient increase, respectively, in the cellular GSSG levels with maximal values that were detectable already after the initial 5 min of incubation (Fig. 3b, c) which was followed by a slow but significant extracellular accumulation of GSSG (Fig. 3e, f). For incubations with 100 µM menadione, almost all the detectable GSx in cells and media represented GSSG (Fig. 3c, f, i), while for cells that had been exposed to 30 µM menadione only around 10% of the cellular GSx but 60% of the extracellular GSx accounted for GSSG (Fig. 3c, f, i). As an incubation with 100 µM menadione for more than 2 h severely impaired membrane integrity (Figs. 1, 2), the percental contribution of GSSG in the GSx values determined was only presented for the initial 2 h of the incubation (Fig. 3c, f, i).

A more detailed analysis of the concentration-dependent potential of menadione to affect the metabolism of

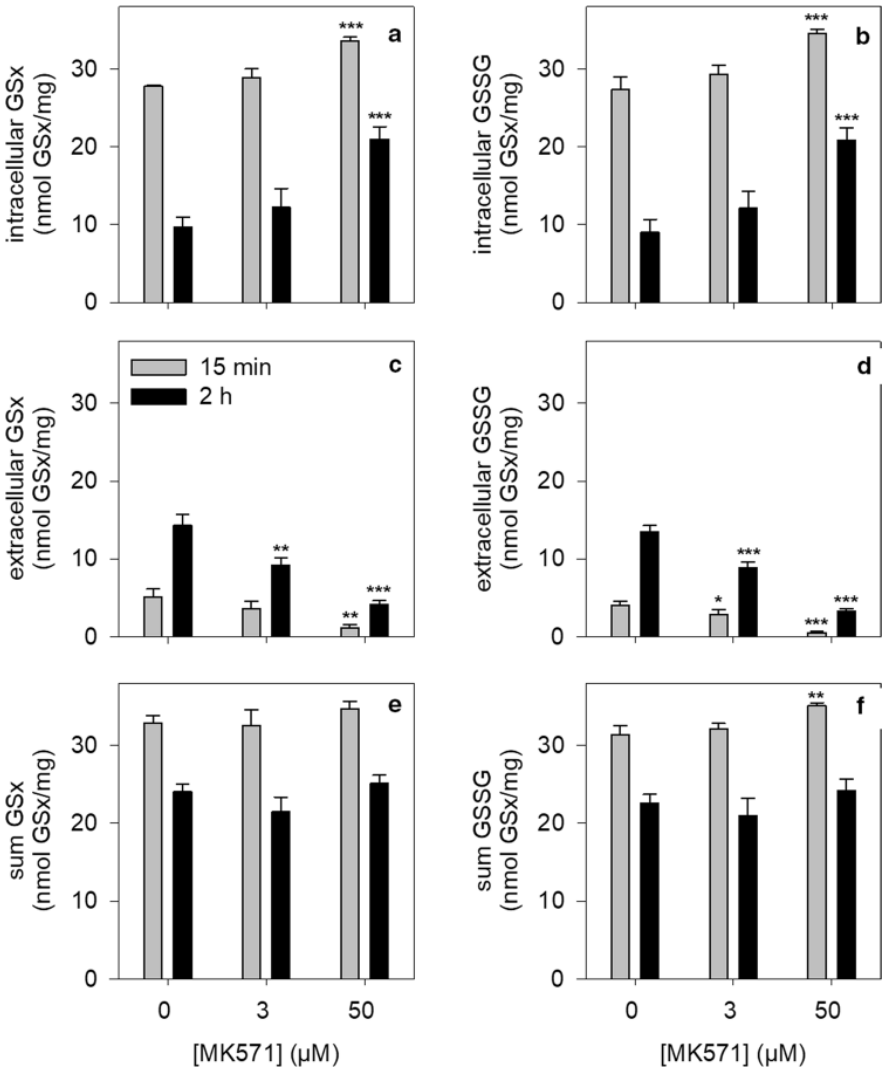


Fig. 6 Inhibition of GSSG export by MK571 in menadione-treated astrocytes. The cultures were incubated for 15 min or 2 h with 100 μM menadione in the absence (0 μM) or the presence of 3 or 50 μM MK571 and the intracellular and extracellular contents of GSx (**a, c**) and GSSG (**b, d**) were determined. In addition, the sum of intra- and extracellular GSx (**e**) and GSSG (**f**) was calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 43 ± 4 nmol/mg, the initial cellular GSSG content 1 ± 0 nmol GSx/mg and the initial protein content 125 ± 19 μg/well. None of the conditions caused any significant increase in extracellular LDH activity (data not shown). The significance of differences compared to the values obtained for the respective control condition (treatment without MK571) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

cultured astrocytes was performed for incubation periods of 15 min and 2 h (Fig. 4). High concentrations of menadione (100–200 μM) caused already within the initial 15 min of incubation a disappearance of cellular (Fig. 4a) and total GSx (Fig. 4e) from the cultures as well as a rapid appearance of GSSG in the cells (Fig. 4b), while little amounts of extracellular GSx and GSSG were determined after 15 min incubation (Fig. 4c, d). However, after 2 h of incubation the cells were almost completely depleted of GSx and GSSG after incubation with menadione in concentrations above 30 μM (Fig. 4a, b) and large amounts of GSSG were determined in the medium (Fig. 4c, d). None of the conditions applied significantly increased extracellular LDH activity (Fig. 4g), demonstrating that viable cells had been investigated. After 2 h of incubation with menadione in concentrations of 50 μM or higher a significant decline by about 70% in the accumulation of extracellular lactate was observed, while after the initial 15 min of incubation no alteration in lactate release was observed even for cultures that had been exposed to 200 μM menadione (Fig. 4h).

Reversibility of the Menadione-Induced GSSG Accumulation

To test for the potential of menadione-treated astrocytes to regenerate the initial very low cellular GSSG to GSx ratio, the cultures were pre-incubated with 100 μM menadione for 5 min. Subsequently, the menadione-containing IB was removed and the cells were incubated for 5 min either in fresh menadione-containing IB or in menadione-free IB. During the short pre-incubation with menadione cellular GSH was rapidly oxidized to GSSG as demonstrated by the observation that the

GSx values determined represented exclusively GSSG (Fig. 5). This high ratio of GSSG to GSx was maintained in cells that were incubated for additional 5 min with menadione (Fig. 5). In contrast, 5 min after removal of menadione cellular GSSG accounted only to 4% of the GSx values determined (Fig. 5), demonstrating rapid reduction of cellular GSSG after removal of menadione.

Inhibition of Menadione-Induced GSSG Export by MK571

Cellular GSSG is exported from viable astrocytes by Mrp1 [19, 21]. To investigate whether the extracellular GSSG accumulation observed for menadione-treated astrocytes is mediated by Mrp1, the Mrp1-inhibitor MK571 [17, 19] was applied. After application of 100 µM menadione for 15 min or 2 h the viability of the cells was not affected as demonstrated by the absence of any increase in extracellular LDH activity (data not shown) and the cellular and extracellular GSx values represented almost exclusively GSSG for all conditions investigated (Fig. 6). The presence of MK571 lowered in a concentration-dependent manner already during the initial 15 min of incubation with 100 µM menadione the loss in cellular GSx and GSSG (Fig. 6a, b) and the extracellular accumulation of GSx and GSSG (Fig. 6c, d). The extent of the inhibitory effect of MK571 on GSSG export was found more pronounced after 2 h of incubation (Fig. 6a–d). In contrast, neither the sum of cellular plus extracellular GSx or GSSG nor the time-dependent menadione-induced disappearance of around 30% of the initial cellular GSx content between 15 min and 2 h of incubation was affected by the presence of MK571 (Fig. 6e, f).

ROS Production in Menadione-Treated Astrocytes

Menadione has been reported to cause ROS formation in cells [39] and cultured astrocytes [29, 30]. In order to investigate whether also under the conditions used here the application of menadione causes rapid ROS production in astrocytes, the presence of ROS was detected for the cells by rhodamine123 staining. After incubation of cultured astrocytes in the absence of menadione only little rhodamine123 staining was observed (Fig. 7b), while already after a 5 min exposure of the cells to menadione a concentration-dependent increase in the number of ROS-positive cells was observed (Fig. 7f, k, o, s) with a ROS staining detectable for the majority of cells in cultures that had been treated with 100 µM menadione (Fig. 7s).

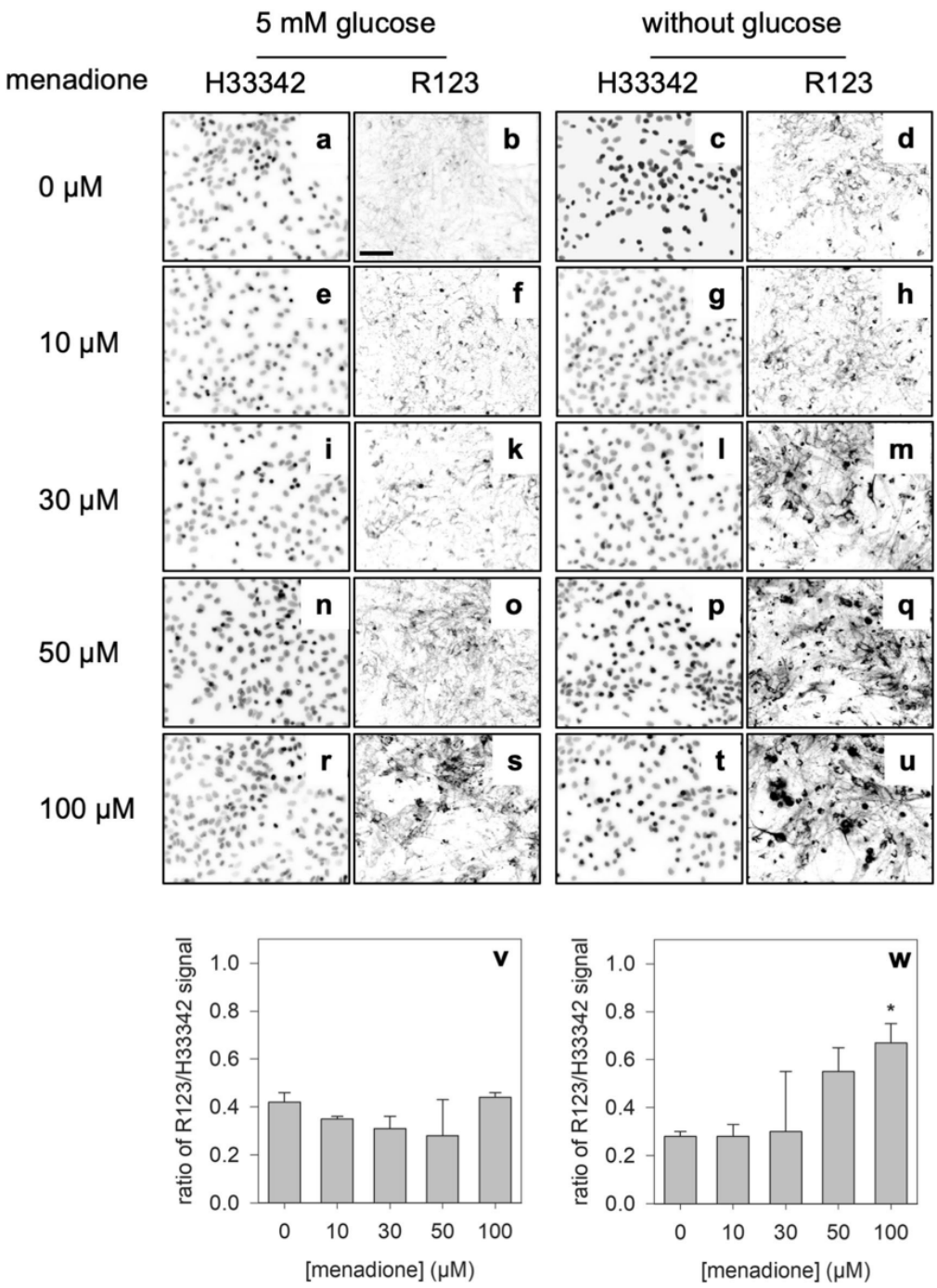
Modulation of Menadione-Induced Formation of ROS and GSSG by the Absence of Glucose

Availability of glucose is important to regenerate via the pentose phosphate pathway NADPH that is required for antioxidative defence [40, 41]. To test whether the menadione-mediated ROS generation and GSH oxidation may be affected by the absence or the presence of glucose and its metabolites, the cells were deprived of intracellular glucose by pre-incubation for 20 min in glucose-free IB (or as glucose control in IB containing 5 mM glucose) and were then incubated with 100 µM menadione in the absence or the presence of 5 mM glucose.

Glucose deprivation did to some minor extent increase the number of ROS-positive cells (Fig. 6b, d) but did not affect the basal cellular and extracellular levels of GSx and GSSG (Fig. 8) in cultures that had not been exposed to menadione. Application of menadione to glucose-deprived and glucose-fed astrocytes caused a concentration-dependent appearance of ROS (Fig. 7) and a concentration-dependent accumulation of intracellular and extracellular GSSG (Fig. 8). However, in glucose-deprived cells the ROS production was found more pronounced (Fig. 7h, m, q, u) than in glucose-fed cells (Fig. 7f, k, o, s). For example, ROS production was already clearly detectable for incubations with 30 µM menadione in glucose-deprived cells (Fig. 7m), but not in glucose-fed cells (Fig. 7k). Also, the quantified and normalized intensity of the ROS staining was found increased with the concentration of menadione applied in glucose-deprived cells (Fig. 6w), but not in glucose-fed cells (Fig. 6v). Similarly, 50 µM or 100 µM of menadione had to be applied to glucose-fed cells to cause a strong increase in cellular GSSG levels, while already a treatment with 10 µM menadione was sufficient to significantly increase cellular and extracellular contents of GSSG in glucose-deprived cells (Fig. 8). The effects observed for a treatment with menadione on the cellular and extracellular levels of GSx and GSSG were for almost every concentration of menadione applied significantly stronger for glucose-deprived cells compared to the respective incubation in the presence of glucose (Fig. 8).

Test for an Involvement of NQO1 in the Menadione-Induced GSH Oxidation

Menadione is a substrate of the enzyme NQO1 [4]. In order to test whether NQO1 is involved in the observed menadione-induced GSH oxidation in astrocytes, the consequences of a menadione exposure to astrocytes in the absence or the presence of the highly potent NQO1 inhibitor dicoumarol [5] was investigated. The presence of 30 µM dicoumarol did not prevent but rather slightly increased the menadione-induced appearance of GSSG in the cultures and did also not prevent the cell toxicity observed after 4 h of menadione treatment (Table 1). Also, the rapid occurrence of ROS in menadione-treated astrocytes was not prevented by the presence of dicoumarol (data not shown). However, the presence of dicoumarol for 15 min caused an increase in the cellular levels of GSx



and GSSG and partially prevented the acute (15 min) extracellular accumulation of GSSG (Table 1), consistent with the recently described potential of dicoumarol to inhibit Mrp1-mediated transport processes [32]. The high extracellular contents of GSx and GSSG determined after 4 h of incubation for both conditions are most likely a consequence of a severely impaired membrane integrity, as demonstrated by the elevated activity of extracellular LDH determined for incubations with menadione in the absence or the presence of dicoumarol (Table 1).

◂**Fig. 7** Effects of the absence or the presence of glucose on the menadione-induced ROS formation. Astrocyte cultures were pre-incubated for 20 min without or with 5 mM glucose and then incubated without or with the indicated concentrations of menadione for 5 min in the absence or the presence of 5 mM glucose. After the incubation, the presence of ROS was visualized by monitoring rhodamine123 (R123) fluorescence. Hoechst 33342 staining was applied to visualize the total number of cell nuclei present. The scale bar in panel b represents 50 µm and applies to all panels. Panels v and w show the relative R123 fluorescence (ratio of the R123–H33342 fluorescence intensity signals) calculated for cells that had been incubated in the presence of 5 mM glucose (v) or in the absence of glucose (w). The quantitative data are means ± SD of results that were obtained in experiments performed on three independently prepared cultures. The significance of differences compared to the values obtained for the respective control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$)

Discussion

Exposure of cultured astrocytes to menadione caused a rapid concentration-dependent increase in cellular ROS and GSSG within minutes that was followed by an impairment of cellular lactate production and finally by a loss in cell membrane integrity. Menadione has previously been applied to cultured astrocytes to induce oxidative stress [26–28]. The menadione-induced ROS production and toxicity observed in our study for cultured astrocytes that had been exposed to high concentrations of menadione is consistent with literature data [29–31]. Menadione is a membrane-permeable molecule that easily penetrates the intact cell membrane of viable astrocytes [42] and leads to rapid ROS formation in the cells. This ROS formation is likely to cause oxidative stress in the cells by oxidation of cellular molecules such as GSH even if the cell membrane is intact, consistent with the reported early damage of mitochondrial DNA in intact astrocytes after menadione exposure [28, 43].

Application of 100 µM menadione caused a rapid formation of ROS and a rapid oxidation of GSH to GSSG in the cells. As GSH is able to reduce various ROS which leads to the generation of GSSG [6, 8, 10], it is assumed that the rapid accumulation of GSSG in astrocytes after application of higher concentrations of menadione is the direct consequence of GSH-mediated ROS detoxification. An impairment of antioxidative enzymes by menadione application such as superoxide dismutase and glutathione peroxidase has not been found for menadione-treated astrocytes [43]. Also, a potential contribution of an inhibition of glutathione reductase in the rapid accumulation of cellular GSSG in menadione-treated cells appears unlikely, as the initial high GSH to GSSG ratio of untreated astrocytes was fully restored within 5 min after removal of menadione following a 5 min exposure to 100 µM menadione.

The extent of adverse consequences of a menadione treatment of cultured astrocytes depends strongly on the concentration of menadione applied. While the cells could deal very well with an exposure to menadione in concentrations of up to 30 µM, the application of 100 µM menadione for more than 2 h severely compromised cell viability. The low levels of ROS production and cellular GSSG accumulation as well as the low toxic potential of menadione in concentrations of up to 30 µM is most likely a consequence of the highly efficient antioxidative defence system of astrocytes [6, 44] which prevented the accumulation of detectable amounts of ROS and a substantial cellular GSSG accumulation under the conditions investigated. This view is supported by the observation that menadione caused already in concentrations of 10 µM detectable ROS production and substantial GSSG oxidation in glucose-deprived astrocytes, consistent with the need of astrocytes for pentose-phosphate pathway (PPP)-derived NADPH for efficient GSSG reduction via glutathione reductase [41, 45].

The menadione-mediated ROS production in astrocytes may involve a direct chemical GSH oxidation by menadione, but also the one-electron reduction of menadione by cytochrome P450 monooxygenases to the unstable semiquinone that causes subsequently cellular ROS production [4, 29, 46]. In contrast, the cytosolic enzyme NQO1 which catalyses the obligatory two-electron reduction which avoids the formation of a semiquinone radical [4, 5] appears to be not involved in the menadione-induced ROS production, as the NQO1 inhibitor dicoumarol was unable to prevent ROS formation and GSSG accumulation in menadione-treated astrocytes. However, it should be stressed here that dicoumarol lowered the export of GSSG from menadione-treated astrocytes consistent with recent literature data [32], demonstrating that dicoumarol cannot be considered anymore as a selective inhibitor of NQO1.

An additional consequence of an application of menadione in concentrations above 30 µM was a disappearance of detectable GSx (sum of GSx in cells plus medium) from the cultures that accounted after 2 h of incubation to around 40% of the initial cellular levels of GSx. This loss in GSx is consistent with recent literature data [32] and is most likely caused by the chemical or enzyme-catalyzed formation of a menadione-GSH conjugate [2, 24] that cannot be detected by the enzymatic cycling assay used for the quantification of GSx.

Application of menadione in a concentration of 100 µM caused a rapid and transient accumulation of GSSG in astrocytes. Maximal cellular GSSG values were found already after 5 min of incubation and the cellular GSSG accounted after 5 min to around 70% of the cellular GSx content, demonstrating that under the conditions used glutathione reductase was not able to immediately reduce the GSSG generated from GSH during ROS detoxification. The loss in cellular GSSG during the subsequent longer incubation was accompanied by the accumulation of GSSG in the incubation medium of viable cells, suggesting that the intracellular

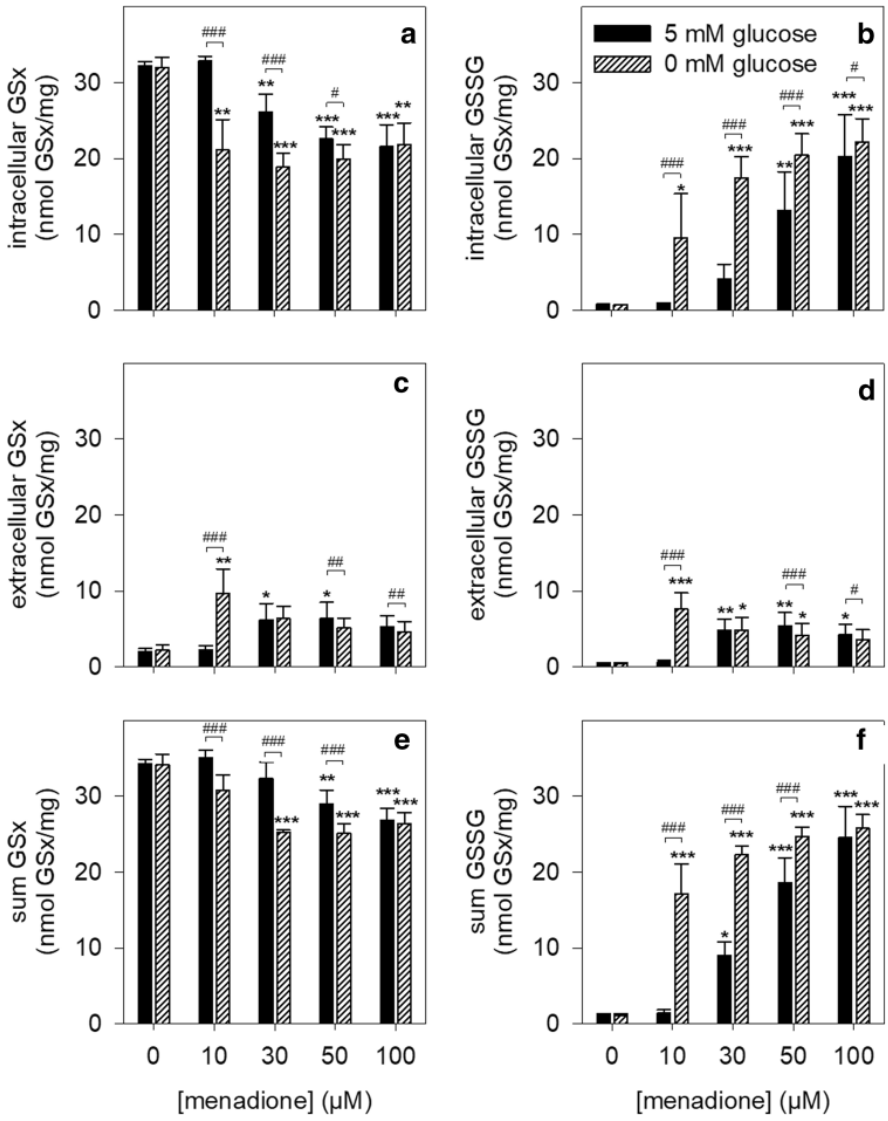


Fig. 8 Effects of the absence or the presence of glucose on the menadione-induced GSSG formation. Astrocyte cultures were pre-incubated for 20 min without or with 5 mM glucose and then incubated without or with 10, 30, 50 or 100 μM menadione for 15 min in the absence or the presence of glucose. Afterwards, the intracellular and extracellular contents of GSx (**a, c**) and GSSG (**b, d**) were determined. In addition, the sum of intra- plus extracellular GSx (**e**) and GSSG (**f**) were calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 36 ± 4 nmol/mg, the initial cellular GSSG content 1 ± 0 nmol GSx/mg and the initial protein content 125 ± 19 μg/well. None of the conditions caused any significant increase in extracellular LDH activity (data not shown). The significance of differences compared to the values obtained for the respective control condition (treatment without menadione) was analysed by ANOVA and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Differences between values obtained for incubations in the absence and in the presence of glucose were analysed by a paired *t* test and the level of significance is indicated by hashes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$)

GSSG was exported from the cells via a transporter. Indeed, MK571 and dicoumarol, known inhibitors of Mrp1-mediated transport processes [17, 21, 32] inhibited the loss of cellular GSSG and the extracellular accumulation of GSSG in menadione-treated astrocyte cultures, suggesting that Mrp1 is mediating the observed GSSG export. Substantial extracellular GSSG accumulation was already observed for astrocytes that had been exposed to 30 μM menadione, although only little cellular accumulation of GSSG was detected for this condition which did not exceed 10% of the cellular GSx content. However, as the GSH concentration in cultured astrocytes is around 8 mM [7] the intracellular GSSG concentration will be around 400 μM even if GSSG accounts for only 10% of the cellular GSx concentration. At this GSSG concentration efficient export of GSSG via Mrp1 has to be expected, as this transporter has a K_M value for its substrate GSSG of 93 μM [18].

Menadione in a concentration above 30 μM lowered also the glycolytic lactate production of cultured astrocytes. As this effect was only observed after more than 15 min of incubation, the lowered lactate production is likely to be a consequence of the initial rapid ROS formation and GSSG accumulation in menadione-treated astrocytes. A reduction in the rate of glycolytic lactate production has also been reported for astrocytes that were exposed to chronic hydrogen peroxide stress, a condition that also caused a rapid accumulation of GSSG in astrocytes [36]. The high ratio of cellular GSSG to GSH found under such stress conditions is likely

Table 1 Modulation by dicoumarol of the menadione-mediated effects on astrocytes

Parameter investigated	Units	Menadione		Menadione + dicoumarol	
		15 min	4 h	15 min	4 h
Extracellular LDH	% Initial cellular activity	0.9 ± 0.8	30.9 ± 6.2	0.5 ± 0.4	24.6 ± 9.7
Intracellular GSx	nmol GSx/mg	30.3 ± 1.8	1.0 ± 0.5	37.7 ± 0.8$^{\#\#}$	2.4 ± 0.1$^{\#}$
Intracellular GSSG		30.7± 6.9	1.5 ± 0.5	40.6 ± 6.0$^{\#}$	2.9 ± 0.3$^{\#}$
Extracellular GSx		5.9 ± 0.6	21.2 ± 1.9	2.0 ± 0.5$^{\#}$	22.5 ± 4.3
Extracellular GSSG		5.3 ± 0.3	19.9 ± 2.1	1.9 ± 0.7$^{\#}$	22.7 ± 5.0
Sum GSx		36.2 ± 1.2	22.1 ± 2.3	39.7 ± 1.1$^{\#\#}$	24.9 ± 4.3
Sum GSSG		36.0 ± 6.6	21.4 ± 2.4	42.5 ± 6.5$^{\#}$	25.6 ± 5.2

Cultured astrocytes were incubated with 100 µM menadione in the absence or the presence of 30 µM dicoumarol for 15 min or for 4 h before the extracellular LDH activity as well as the intracellular and extracellular contents of GSx and GSSG were determined. In addition, the sum of intracellular plus extracellular GSx and of intracellular plus extracellular GSSG were calculated. The data shown represent means ± SD of values obtained in experiments performed on three independently prepared cultures. The initial cellular GSx content of the cultures was 49 ± 4 nmol/mg and the protein content was 121 ± 15 µg/well. The initial cellular LDH activity (100%) was determined as 2163 ± 150 nmol/(min × mg). The significance of differences between data obtained for treatments without and with dicoumarol was analysed for the respective time points by the paired *t* test and the level of significance is indicated by hashes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$)

to trigger a redox switch which re-directs the metabolism of glucose from glycolysis into the oxidative parts of the PPP in order to facilitate re-generation of NADPH that is needed for antioxidative defence [41, 47, 48].

Oxidative stress is frequently applied to cultured brain cells by exposure to hydrogen peroxide [49, 50]. As especially astrocytes but also other brain cell types in culture are very efficient to dispose applied hydrogen peroxide [44] the extent of intracellular GSSG accumulation after application of a peroxide is low and is restricted to the time period required for the cellular detoxification system to remove the applied peroxide which immediately enables glutathione reductase to regenerate the initial very high GSH to GSSG ratio. In contrast, during exposure to 100 µM menadione a rapid and extensive ROS formation was observed that was accompanied with a quick and extensive oxidation of the cellular GSH to GSSG within minutes. As the redox cycling of menadione has the potential to generate continuously ROS for at least 1 h [26], the ROS-mediated GSH oxidation continues and cellular GSSG accumulation cannot be efficiently prevented by glutathione reductase. This makes an cellular export of GSSG via Mrp1 a preferred option to lower the cellular concentration of GSSG and thereby the high ratio of GSSG to GSH. Thus, the menadione-induced rapid and extensive increase in cellular GSSG concentration can be used as valuable experimental approach to investigate consequences of oxidative stress on the GSH metabolism in astrocytes and in particular to investigate GSSG export processes as recently described [32].

In conclusion, application of menadione to cultured astrocytes triggers a rapid concentration-dependent ROS formation and GSH oxidation which is followed by Mrp1-mediated export of GSSG. This confirms the strong potential of menadione to cause oxidative stress in cultured brain cells by a NQO1-independent process. The experimental setup used allows to investigate various aspects of menadione-induced oxidative stress and defines the conditions suitable to study ROS formation, GSH oxidation and Mrp1-mediated GSSG export as well as ROS-induced toxicity in cultured brain astrocytes after menadione exposure.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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2.3 Publication 2

Beta-lapachone induces severe oxidative stress in rat primary astrocyte cultures that is prevented by the NQO1 inhibitor dicoumarol.

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Neurochemical Research, manuscript accepted for publication (2020)

Contributions of Johann Steinmeier:

- Preparation of schemes as figures 1 and 10
- Data shown in figures 6, 7, 8 and 9
- 90% of preparation of the first manuscript draft

- Data shown in figures 2, 3, 4 and 5 were obtained by Sophie Kube for her master thesis which was co-supervised by Johann Steinmeier

β-lapachone induces acute oxidative stress in rat primary astrocyte cultures that is terminated by the NQO1-inhibitor dicoumarol

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Abstract

β–lapachone (β-lap) is reduced in tumor cells by the enzyme NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) to a labile hydroquinone which spontaneously reoxidises to β-lap, thereby generating reactive oxygen species (ROS) and oxidative stress. To test for the consequences of an acute exposure of brain cells to β-lap, cultured primary rat astrocytes were incubated with β-lap for up to 4 h. The presence of β-lap in concentrations of up to 10 µM had no detectable adverse consequences, while higher concentrations of β-lap compromised the cell viability and the metabolism of astrocytes in a concentration- and time-dependent manner with half-maximal effects observed for around 15 µM β-lap after a 4 h incubation. Exposure of astrocytes to β-lap caused already within 5 min a severe increase in the cellular production of ROS as well as a rapid oxidation of glutathione (GSH) to glutathione disulfide (GSSG). The transient cellular accumulation of GSSG was followed by GSSG export. The β-lap-induced ROS production and GSSG accumulation were completely prevented in the presence of the NQO1 inhibitor dicoumarol. In addition, application of dicoumarol to β-lap-exposed astrocytes caused rapid regeneration of the normal high cellular GSH to GSSG ratio. These results demonstrate that application of β-lap to cultured astrocytes causes acute oxidative stress that depends on the activity of NQO1. The sequential application of β-lap and dicoumarol to rapidly induce and terminate oxidative stress, respectively, is a suitable experimental paradigm to study consequences of a defined period of acute oxidative stress in NQO1-expressing cells.

Keywords: astrocytes; dicoumarol; GSSG; NQO1; oxidative stress

Introduction

The quinone beta-lapachone (β-lap, clinical names: ARQ761 or ARQ501) has been extracted from the bark of the lapacho tree and is known to have various beneficial effects (Kung *et al.* 2014, Bermejo *et al.* 2017). For example, it has frequently been applied in anti-cancer studies on cells and tissues, targeting for example prostate cancer (Don *et al.* 2001), pancreatic cancer (Ough *et al.* 2005), lung cancer (Jeon *et al.* 2015), breast cancer (Yang *et al.* 2017), melanoma (Bang *et al.* 2016) or astrocyte-like glioma (Xu *et al.* 2016). The proposed mechanism of the antitumor action is the activation of β-lap by NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1, EC 1.6.99.2) (Fig. 1), which catalyses the obligatory two-electron reductions of quinones (Dinkova-Kostova and Talalay 2010, Siegel *et al.* 2012a). NQO1-mediated catalysis is widely regarded as beneficial, as it avoids an undesirable one-electron reduction that is directly associated with radical formation and oxidative stress (Dinkova-Kostova and Talalay 2010, Klotz *et al.* 2014, Sies *et al.* 2017) and alleviates clearance by phase II enzymes (Dinkova-Kostova and Talalay 2010). However, the hydroquinone form of β-lap (β-lapachol, Fig. 1) that is generated by NQO1-mediated reduction is labile and auto-oxidizes quickly in two distinct one-electron steps (Siegel *et al.* 2012b, Kung *et al.* 2014), thereby starting a futile cycle that regenerates the quinone β-lap by producing intracellular ROS which lead to oxidative stress and cell toxicity (Bermejo *et al.* 2017, Silvers *et al.* 2017). This NQO1-dependent cycling of β-lap appears to be especially prominent in cancer cells, since such cells are reported to contain higher activities of NQO1 than non-cancerous cells, which supports the potential use of β-lap as an anti-cancer drug (Kung *et al.* 2014, Li *et al.* 2014).

In brain, astrocytes are the first parenchymal cells behind the blood-brain barrier (Verkhratsky *et al.* 2015, Gothwal *et al.* 2018) and are therefore considered as the first line of defence against xenobiotics causing oxidative stress (Dringen *et al.* 2015).

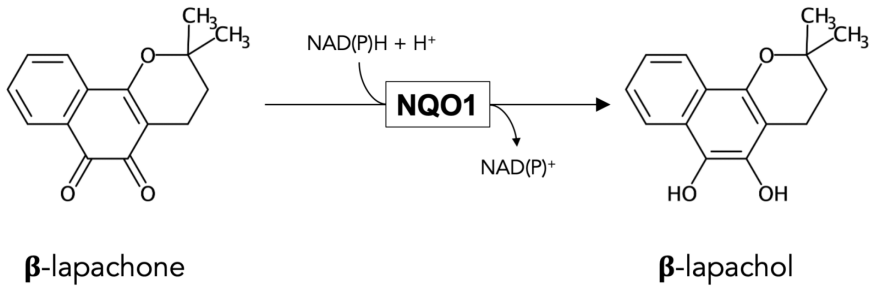

Figure 1: β-lapachone and β-lapachol. β-lapachone (quinone-form) can be reduced in a two-electron reaction to β-lapachol (hydroquinone-form) by NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1).

Astrocytes possess an elaborated antioxidative defence system, that includes high activities of antioxidative enzymes and millimolar concentrations of the isopeptide glutathione (GSH) (Dringen and Hamprecht 1998, Dringen 2000, Bolaños 2016). Glutathione is a crucial antioxidant (Deponte 2013, Dringen *et al.* 2015, Sies *et al.* 2017) that plays a key role in the maintenance of the cellular thiol-redox potential and is substrate of enzymes involved in the defence of cells against oxidative stress and xenobiotics (Dringen *et al.* 2015). The product of the oxidation of GSH by glutathione peroxidases (GPx, EC 1.11.1.9) is glutathione disulfide (GSSG), which is quickly recycled in viable cells to GSH by the NADPH-consuming glutathione reductase (GR, EC 1.8.1.7) (Dringen *et al.* 2015, Couto *et al.* 2016). In addition, GSH can be conjugated to xenobiotics by glutathione-*S*-transferases to GSH-conjugates (Dringen *et al.* 2015). In unstressed astrocytes, hardly any GSSG is detectable, but several compounds have been described to induce GSH oxidation to GSSG which is subsequently exported from the cells, including peroxides (Dringen and Hamprecht 1997), catecholamines (Hirrlinger *et al.* 2002a) as well as quinones such as menadione (Raabe *et al.* 2019, Steinmeier and Dringen 2019). Depletion of cellular GSH in astrocytes was also reported for cells that had been exposed to alkylating substances like iodoacetate (Schmidt and Dringen 2009), 3-bromopyruvate (Ehrke *et al.* 2015) or dialkyl-fumarates (Schmidt and Dringen 2010). In cultured astrocytes, the export of GSH, GSSG and GSH-conjugates is mediated by the ATP-dependent multidrug resistance protein 1 (Mrp1) (Hirrlinger *et al.* 2001, Hirrlinger *et al.* 2002b, Minich *et al.* 2006, Waak and Dringen 2006, Tulpule *et al.* 2012).

So far little information is available on the consequences of an exposure of brain cells to β-lap. Chronic exposure to 1 µM β-lap has been reported to induce in primary rat astrocytes the expression of protective and antioxidative enzymes (e.g. NQO1, catalase), to increase cellular GSH contents and to protect the cells against hydrogen peroxide-induced oxidative stress (Park *et al.* 2016). These results are consistent with a recent report on the neuroprotective potential of β-lap in a MPTP-induced Parkinson's disease mouse model which involves the upregulation of Nrf2-controlled pathways in astrocytes (Park *et al.* 2019). Even lower concentrations of β-lap than 1 µM have been reported to activate glutamate dehydrogenase and to attenuate iodoacetate induced toxicity in cultures of cortical neurons or astrocytes (Kim *et al.* 2017). Moreover, exposure of rat primary microglia to β-lap lowered nitrite and ROS levels and increased the expression of NQO1 (Lee *et al.* 2015).

Astrocytes have been reported to contain substantial activity of NQO1 (Park *et al.* 2016, Ehrke *et al.* 2020) and should therefore encounter NQO1-mediated

ROS production and oxidative stress after application of β-lap. Here we report that acute exposure of cultured astrocytes to low micromolar concentrations of β-lap caused rapid cellular ROS formation and GSH oxidation which is followed by GSSG export and a delayed impairment in cell metabolism and cell viability. All these adverse effects of a β-lap application to astrocytes were completely prevented by the NQO1 inhibitor dicoumarol, demonstrating that the presence of this enzyme makes cultured astrocytes sensitive towards β-lap. In addition, the dependence of β-lap-induced oxidative stress on the activity of NQO1 makes the sequential application of β-lap and dicoumarol a suitable experimental system to rapidly induce (application of β-lap) and terminate (addition of dicoumarol) an acute oxidative stress condition in cultured NQO1-expressing cells.

Material and methods

Material

β-lap (ab141097) was purchased from Abcam (Berlin, Germany) and dicoumarol (M1390) from Sigma-Aldrich (Steinheim, Germany). Dulbecco's modified Eagle's medium (DMEM with 25 mM glucose) and penicillin/streptomycin solution was obtained from Gibco (Darmstadt, Germany) and fetal calf serum from Biochrom (Berlin, Germany). Bovine serum albumin (BSA), NADPH, NADH and sulfosalicylic acid (SSA) were from AppliChem (Darmstadt, Germany) and the enzyme glutathione reductase was purchased from Roche Diagnostics (Mannheim, Germany) and from Sigma-Aldrich (Steinheim, Germany). All other chemicals of the highest purity available were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Dojindo (Munich, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany) or Riedel-de Haën (Seelze, Germany). Sterile cell culture plates and non-sterile normal and black 96-well microtiter plates were from Sarstedt (Nümbrecht, Germany).

Astrocyte cultures

Primary astrocyte cultures were prepared from whole brains of new-born Wistar rats after decapitation as previously described (Tulpule *et al.* 2014). Wistar rats were purchased from Envigo RMS Rossdorf, Germany. Animals were treated in accordance with the animal regulations of the University of Bremen and of the state of Bremen, Germany. For the preparation of primary cultures a special institutional ethical approval was not required. Primary astrocytes cultures contain mainly GFAP-expressing astrocytes and only minor amounts of oligodendrocytes and microglial cells (Hamprecht and Löffler 1985, Tulpule *et al.* 2014). Harvested cells were seeded in a density of 300,000 viable cells in 1 mL of culture medium (90 % DMEM, 10 % FCS, 1 mM pyruvate, 18 U/mL penicillin G and 18 µg/mL streptomycin sulfate) in wells of a 24-well plate and incubated for at least two weeks at 37°C with 10 % CO_2 in the humidified atmosphere of a cell incubator (Sanyo, Japan). Experiments were performed on confluent cultures of an age between 14 and 28 days. The only exception from this was one culture used for one replication of the experiment shown in figure 8, which was 39 days old. Every 7 days and one day before an experiment the culture medium of the cells was renewed.

Cell incubations

Astrocyte cultures were washed with 0.5 mL pre-warmed (37 °C) incubation buffer (IB; 20 mM HEPES, 5 mM D-glucose, 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4, pH 7.4) and incubated at 37 °C with 200 µL IB in the absence or the presence of the other compounds indicated in the figure legends in the humidified atmosphere (without CO_2) in a cell incubator (Sanyo, Japan). After the given incubation periods, media were harvested and the cells were washed with 1 mL ice-cold (4°C) phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) for subsequent analysis of cellular and extracellular components.

Tests for cell viability

The viability of astrocyte cultures after a given treatment was determined by testing for membrane integrity by measuring the extracellular lactate dehydrogenase (LDH, EC 1.1.1.27) activity and by nuclear propidium iodide staining as described previously (Tulpule *et al.* 2014). Extracellular LDH was determined in 10 µL media samples. The activity is given as the percentage of the initial cellular LDH activity of control cells that had been lysed in 200 µL IB containing 1 % (v/v) Triton X-100. For propidium iodide staining the treated cells were washed twice with 1 mL prewarmed (37°C) IB, incubated for 15 min at 37°C with 500 µL propidium iodide staining solution (5 µM propidium iodide plus 10 µM Hoechst (H33342) in IB) and subsequently washed twice with 1 mL IB before fluorescence images were recorded using a Nikon Eclipse TE2000U fluorescence microscope equipped with a DSQiMc camera and the imaging software NIS-Elements (Nikon, Düsseldorf, Germany). Appropriate filter settings were used for the visualization of the staining with propidium iodide (excitation: 510-560 nm; emission: 590 nm; dichromatic mirror: 575 nm) and H33342 (excitation: 330-380 nm; emission: 435-485 nm; dichromatic mirror: 400 nm). For all images identical microscopic setting and image processing was applied.

Lactate and protein quantification

The extracellular accumulation of lactate during the incubation was measured for 10 µL medium samples by a coupled enzymatic assay system containing LDH and glutamate pyruvate transaminase as described previously in detail (Liddell *et al.* 2009, Tulpule *et al.* 2014). The initial protein content of the astrocyte cultures investigated

was determined according to the Lowry method (Lowry *et al.* 1951) using bovine serum albumin as standard protein.

Quantification of glutathione contents

Total glutathione (GSx = amount of GSH plus twice the amount of GSSG) and GSSG were quantified for cell lysates and medium samples by a colorimetric enzymatic cycling assay as previously described in detail (Tulpule *et al.* 2014) which is based on the method originally described by Tietze (1969) . The washed cells were lysed with 200 µL 1 % (w/v) sulfosalicylic acid at 4°C and the lysates were used to determine the contents of GSx and GSSG. Quantification of the extracellular contents of GSx and GSSG was performed for 20 µL of a 1:1 mixture of the harvested medium and 1 % (w/v) sulfosalicylic acid.

Determination of ROS production

For quantification of cellular ROS production, the non-fluorescent dihydrodichlorofluorescein-diacetate ($DCFH_2$-DA) was applied which is trapped within cells by enzymatic deacetylation to the $DCFH_2$ which can be oxidized in cells by ROS to the fluorescent dichlorofluorescein (DCF) (Wan *et al.* 2003). Cultured astrocytes were washed with 1 mL of IB and loaded with the non-fluorescent dye by a 30 min incubation with 200 µL 50 µM $DCFH_2$-DA in IB at 37 °C. Subsequently, the cells were washed twice with 1 mL of IB and incubated for 5 min at 37°C with 200 µL IB containing β-lap in the indicated concentrations in the absence or the presence of the NQO1 inhibitor dicoumarol. Finally, the cells were lysed in 400 µL ice-cold (4°C) hypotonic potassium phosphate buffer (20 mM, pH 7.4) on ice for 10 min in the dark and the harvested lysates were centrifuged for 1 min at 12 300 *g*. DCF fluorescence (excitation at 485 nm, emission at 520 nm) was determined for 200 µL of lysate supernatant in wells of a black microtiter plate in a plate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific, Schwerte, Germany).

Determination of extracellular WST1 formazan production

The water-soluble tetrazolium salt 1 (WST1) is a membrane impermeable substance that can be reduced in cell cultures by membrane permeable electron cyclers to form a water-soluble formazan product (Stapelfeldt *et al.* 2017). To test whether β-lap can serve as electron cycler for WST1 reduction, cultured astrocytes were washed once with 1 mL of IB and then incubated in 200 µL of IB containing 5 mM glucose, 20 µM β-lap and 400 µM WST1 in the absence or the presence of other compounds which are listed in the legend of figure 8. After 10 min or 30 min of incubation, 50 µL of incubation solution were harvested, diluted with 150 µL of water in wells of a microtiter plate and the absorbance of the WST1 formazan generated was measured at 450 nm in a plate reader. The concentration of WST1 formazan in the incubation media was calculated from the absorbance by using the extinction coefficient of 35.2 $mM^{-1}x\ cm^{-1}$ (Stapelfeldt *et al.* 2017).

Presentation of data and statistical analysis

The quantitative data presented are means ± standard deviations (SD) of values that were obtained in experiments performed on three independently prepared astrocyte cultures. For analysis of the significance of differences between three or more sets of data analysis of variance (ANOVA) followed by a Bonferroni *post-hoc* test was used, and statistically significant differences are indicated by asterisks which are written in the colours of the respective symbols. p-values above 0.05 were considered as not significant. Statistical analysis was performed using the software GraphPad InStat version 3.10. The images shown for propidium iodide staining of cultured astrocytes are derived form a representative experiment that was reproduced twice on independently prepared astrocyte cultures with similar outcome.

Results

β-lap impairs cell metabolism and cell viability of cultured astrocytes

In order to test whether β-lap has adverse consequences on astrocytes, cultured rat astrocytes were exposed to this compound in concentrations of up to 100 µM for up to 4 h and the membrane integrity as well as the lactate accumulation in the incubation medium were determined. Incubations without or with 10 µM β-lap did not cause any increase in the extracellular LDH activity (Fig. 2a), any alteration in the lactate released from the cultures (Fig. 2b) and no increase in the number of PI-positive cells (Fig. 3g, h, t, u) during incubations for up to 4 h. In contrast, β-lap in concentrations above 10 µM impaired the membrane integrity of astrocytes in a time- and concentration-dependent manner as demonstrated by the gradual increase in the extracellular LDH activity (Fig. 2a) and in the extent of PI staining (Fig. 3). Also the glucose metabolism of astrocytes was affected by β-lap as demonstrated by a concentration-dependent decrease in the accumulation of extracellular lactate which was found significant after

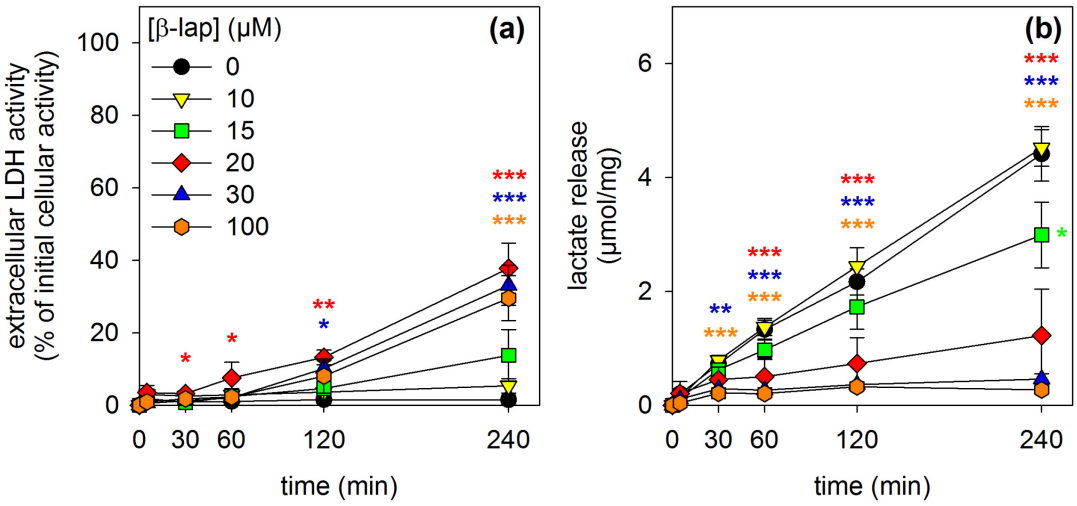

Figure 2: Time- and concentration-dependent effects of β-lap on the viability and the lactate release of primary astrocytes. The cells were incubated with the indicated concentrations of β-lap for up to 240 min. For the indicated time points, the extracellular LDH activity (a) and the extracellular lactate content (b) were determined. The protein content of the cultures was 137 ± 5 µg/well. The data shown are means ± SD of values obtained in 3 experiments performed on independently prepared cultures (n=3). Significant differences (ANOVA) of data compared to the data obtained for control cells (incubation without β-lap) are indicated by asterisks written in the colours of the respective symbols (*p<0.05, **p<0.01, ***p<0.001).

4 h of incubation with 15 µM β-lap, while extracellular lactate accumulation was hardly detectable already after a 30 min exposure to 30 µM or 100 µM β-lap (Fig. 2b).

The further analyses of acute effects of β-lap on cultured astrocytes was restricted to incubation periods of up to 120 min as the membrane integrity of the treated cells was not compromised for these incubation periods (Fig. 3).

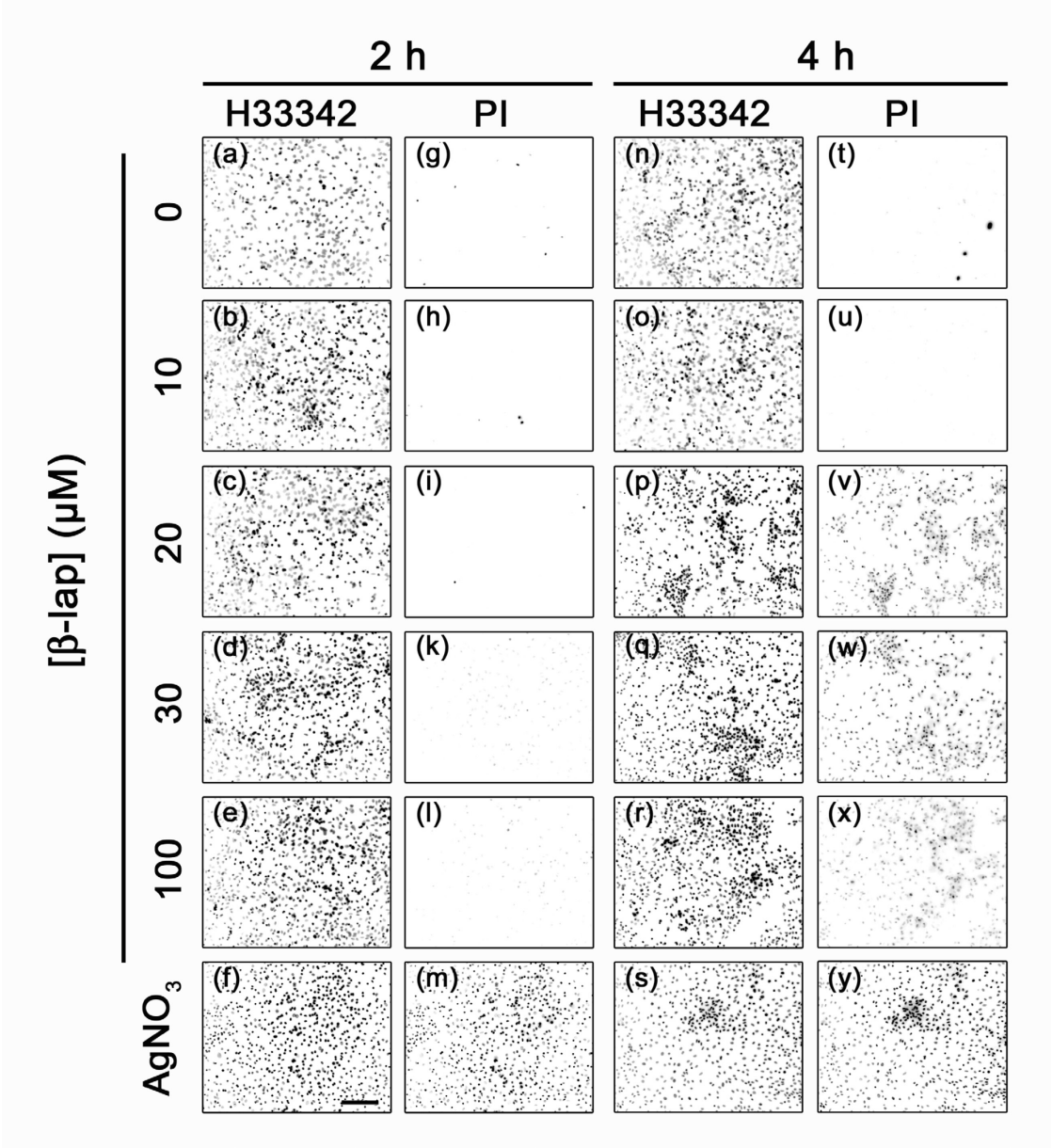

Figure 3: Impairment by β-lap of cell membrane integrity of primary astrocytes. The cells were incubated for 2 or 4 h in IB containing the indicated concentrations of β-lap. As positive control for the loss of cell membrane integrity (Luther *et al.* 2011), cells were incubated with 200 µM (2 h) or 100 µM (4 h) $AgNO_3$. Shown are cell images of astrocytes that had been stained with H33342 and PI after the given β-lap treatment. The scale bar in panel (f) corresponds to 100 µm and applies for all panels. The data shown are from one representative experiment that was repeated twice on independently prepared cultures with similar results.

β-lap induces GSH oxidation and GSSG export

β-lap has been reported to cause oxidative stress in cells (Bey *et al.* 2013). In order to test whether an application of β-lap affects the astrocytic GSH metabolism and the ratio of GSH to GSSG, cultured astrocytes were exposed to β-lap in concentrations of up to 100 µM and the cellular and extracellular contents of GSx and GSSG were determined. During incubation of astrocytes for up to 2 h in the absence of β-lap the cellular GSx content remained almost identical to the initial cellular GSx content (Fig. 4a), a slow increase in the extracellular GSx content was observed (Fig. 4c), the sum of cellular plus extracellular GSx remained constant (Fig. 4e) and only minute amounts of GSSG were found in cells and media (Fig. 4b, d, f). In contrast, already the presence of 10 µM β-lap caused, compared to the control incubations, a time-dependent decrease in the cellular GSx content (Fig. 4a) which was accompanied by an increased extracellular accumulation of GSx (Fig. 4c), as well as a transient appearance of GSSG in the cells within the first 5 min of incubation (Fig. 4b) which was followed by an increased extracellular accumulation of GSSG during the initial 60 min of the incubation (Fig. 4d). Higher concentrations of β-lap than 10 µM further accelerated the loss in cellular GSx, (Fig. 4a), the accumulation of extracellular GSx (Fig. 4c), the appearance of GSSG in cells (Fig. 4b) and the export of GSSG from the cells (Fig. 4d). In addition, with increasing concentration of β-lap, the decline of the initial high GSSG to GSx ratio observed after 5 min of incubation (Fig. 4b) as well as the extracellular GSSG accumulation (Fig. 4d) became slower and a loss in the sum of cellular plus extracellular GSx (Fig. 4e) as well as a strong increase in the sum of cellular plus extracellular GSSG (Fig. 4f) were observed. Almost maximal effects on the GSx and GSSG contents of cultured astrocytes were found for cultured astrocytes that had been exposed to β-lap in a concentration of 20 µM (Fig. 4).

Dicoumarol prevents β-lap-induced GSSG formation

The enzyme NQO1 has been proposed to catalyse a two-electron reduction of β-lap (Siegel *et al.* 2012b) which generates the labile reduction product β-lapachol and subsequently ROS and oxidative stress. In order to test for an involvement of NQO1 in the observed β-lap induced changes in astrocytic viability and GSH metabolism, the NQO1-inhibitor dicoumarol (Ernster *et al.* 1960, Hollander and Ernster 1975) was applied in concentrations of 1 or 30 µM.

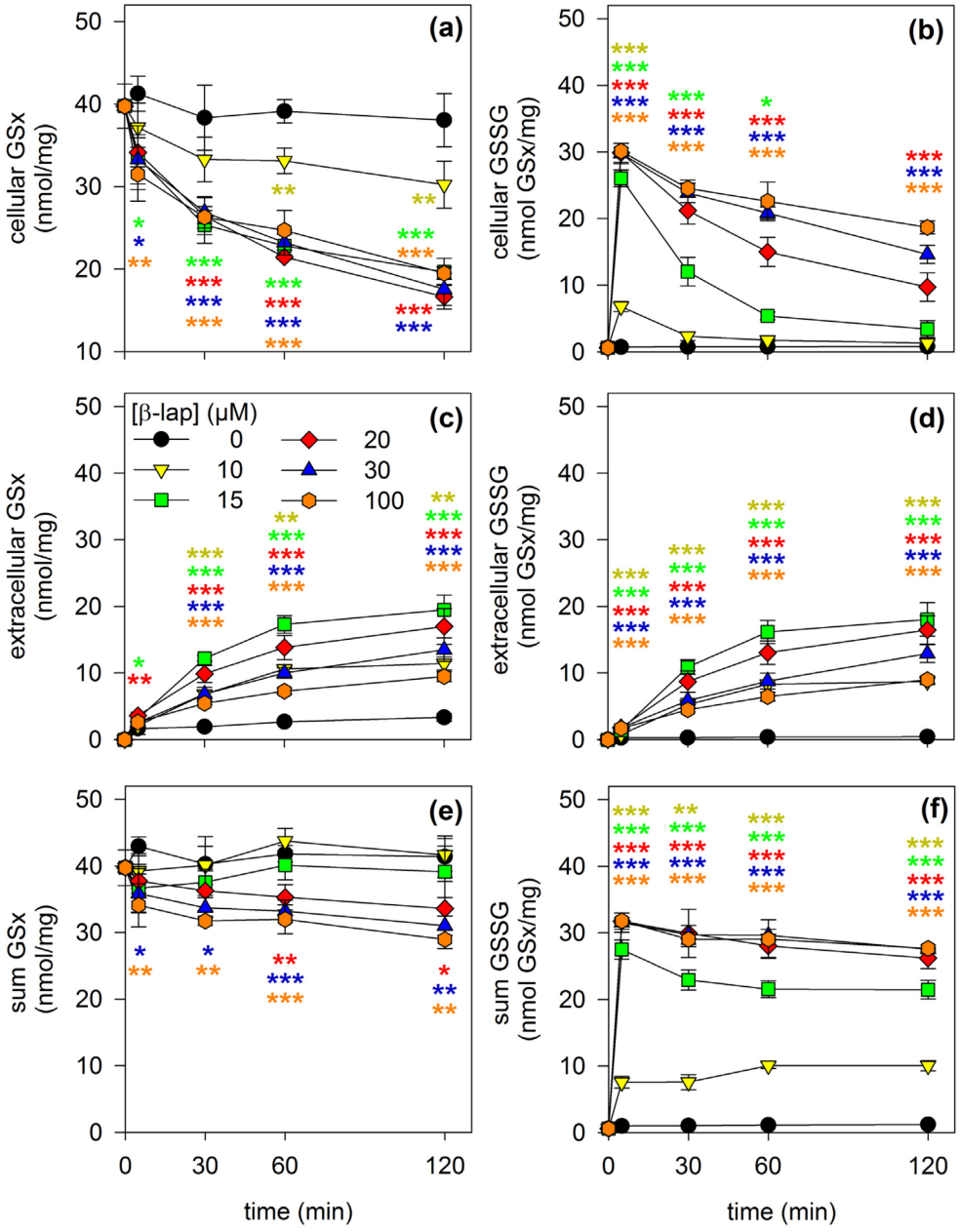

Figure 4: Time- and concentration-dependent effects of a β-lap treatment on the GSx and GSSG contents of astrocyte cultures. The cells were incubated with β-lap in the indicated concentrations for up to 120 min. For the indicated time points the intracellular GSx (a) and GSSG (b) contents, the extracellular GSx (c) and GSSG (d) contents as well as the sum of intra- plus extracellular GSx (e) and GSSG (f) contents of the cultures were determined. The initial specific GSx and GSSG content of the cultures were 40 ± 3 nmol/mg and 1 ± 0 nmol/mg, respectively. The protein content of the cultures was 137 ± 5 µg/well. The data shown are means ± SD of values obtained in 3 experiments on independently prepared cultures (n=3). Significant differences as analysed by ANOVA between the values obtained for a given incubation with β-lap compared with the data for control cells (incubation without β-lap) are indicated by asterisks written in the colours of the respective symbols (*p<0.05, **p<0.01, ***p<0.001).

Incubation of astrocytes with 20 µM β-lap in the absence of dicoumarol affected the cellular and extracellular GSx and GSSG contents as well as the cells viability (Fig. 5) as already described for this condition above (Figs. 2, 4). If 1 µM dicoumarol was present during the incubation of astrocytes with β-lap, the rapid accumulation of cellular GSSG in β-lap-treated cells (88% of GSx after 5 min) was found strongly reduced (19% of GSx after 5 min; Fig. 5b), while the extracellular accumulation of GSSG was not altered compared to the incubation with β-lap alone (Fig. 5d). The sum of cellular plus extracellular GSx was hardly affected by the additional presence of 1 µM dicoumarol (Fig. 5e), while the sum of cellular plus extracellular GSSG values accumulated slowly over the incubation period of 2h to values also found for astrocytes that had been exposed to β-lap alone (Fig. 5f). In addition, the presence of dicoumarol in a concentration of 1 µM prevented the β-lap-induced loss in cell viability (Fig. 5g) and the impaired lactate production (Fig. 5h)

If 30 µM dicoumarol was present during the incubation of astrocytes with β-lap, the cellular GSx content was slightly increased in comparison to the control incubation (Fig. 5a) and the extracellular accumulation of GSx (Fig. 5c), GSSG (Fig. 5d) and the sum of cellular plus extracellular GSx (Fig. 5e) was completely prevented. Importantly, presence of 30 µM dicoumarol also prevented the β-lap-induced transient cellular accumulation of GSSG (Fig. 5b) and LDH release (Fig. 5g) and restored the glycolytic lactate production which was found to be impaired in cells that had been treated with β-lap alone (Fig. 5h). In conclusion, the β-lap-induced impairments of cell viability and glycolytic metabolism was prevented by the presence of either 1 or 30 µM dicoumarol (Fig. 5), while 30 µM of dicoumarol had to be present to abolish the cellular and extracellular accumulation of GSSG in β-lap-induced astrocytes.

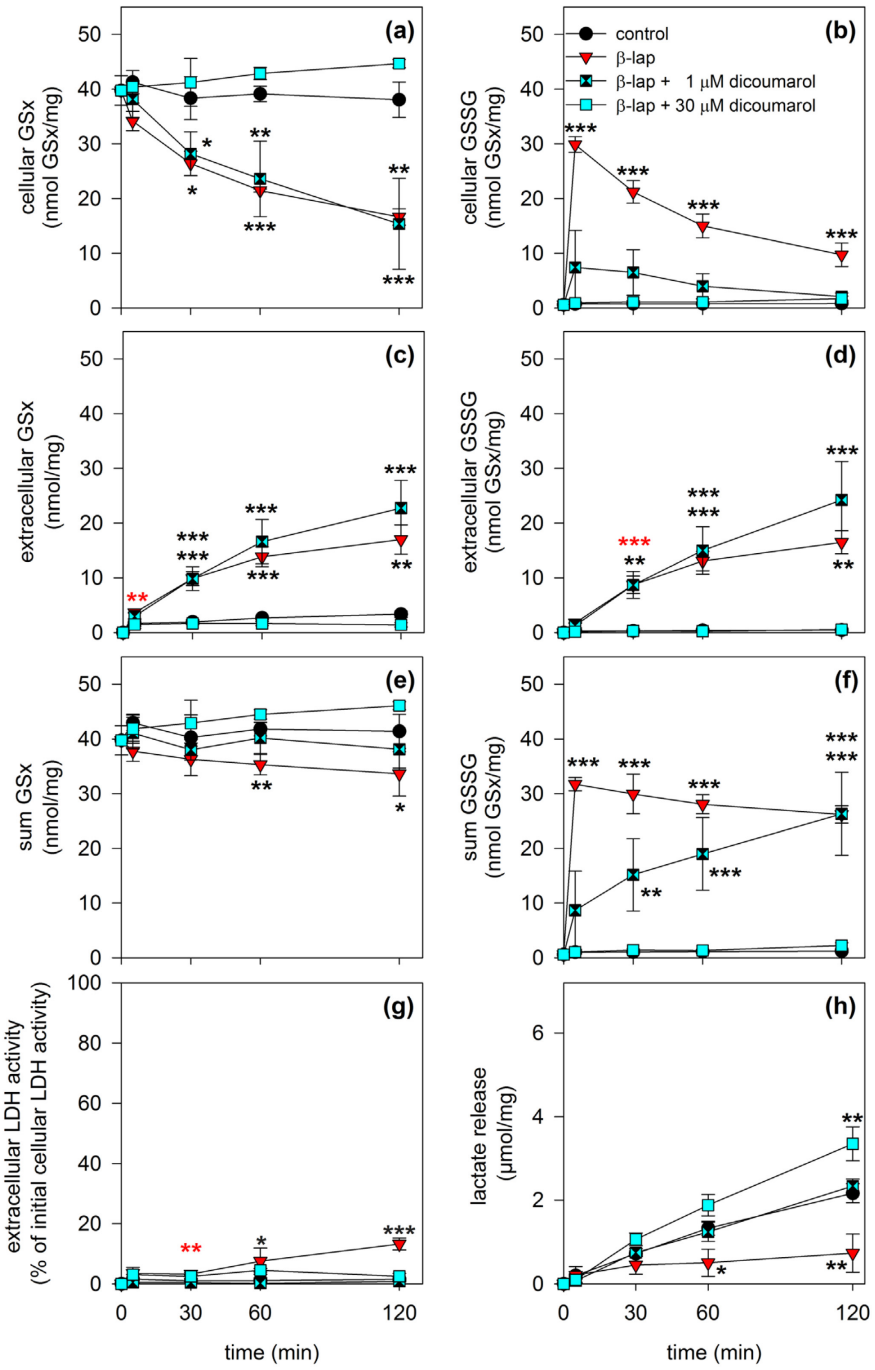

Figure 5: Impact of dicoumarol on the β-lap-induced GSH oxidation in cultured astrocytes. The cells were incubated without (control), with 20 µM β-lap alone, with 20 µM β-lap plus 1 µM dicoumarol or with 20 µM β-lap plus 30 µM dicoumarol for up to 120 min. For the indicated time points the intracellular GSx (a) and GSSG (b) contents, the extracellular GSx (c) and GSSG (d) contents as well as the sum of intra- plus extracellular GSx (e) and GSSG (f) contents of the cultures were determined, and the extracellular LDH activity (g) and the extracellular lactate concentration (h). The data shown are means ± SD of values obtained in 3 experiments on independently prepared cultures (n=3). The initial specific GSx content of the cultures was 40 ± 3 nmol/mg and the initial specific GSSG content was 1 ± 0 nmol/mg. The protein content of the cultures was 137 ± 5 µg/well. The significance of differences (ANOVA) of data compared to the data obtained for control cells is indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001).

β-lap induces ROS formation in astrocytes

To demonstrate ROS formation in β-lap-treated astrocytes, the cultures were loaded with $DCFH_2$-DA before β-lap was applied. Quantification of cellular DCF fluorescence revealed that astrocytes that had been exposed for 5 min to β-lap in concentrations of 20 µM or 100 µM contained DCF contents that were increased by 70 % and 150 %, respectively (Fig. 6) compared to control cells (absence of β-lap). These increases in DCF fluorescence in β-lap-treated astrocytes were completely prevented, if astrocytes had been incubated with β-lap in the presence of 30 µM dicoumarol (Fig. 6).

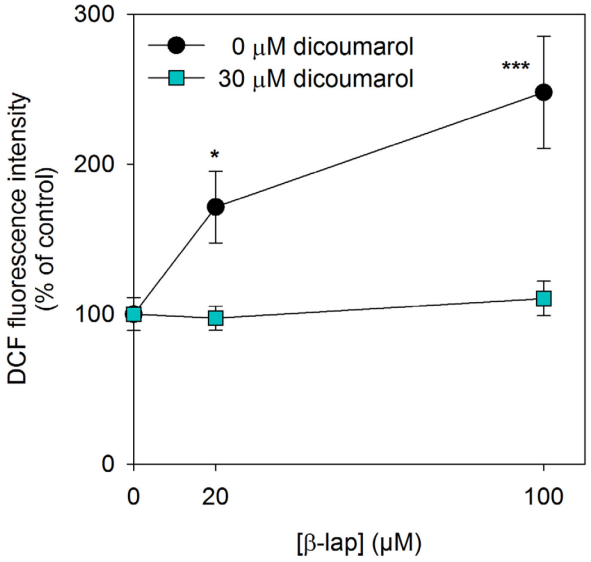

Figure 6: Test for ROS production in β-lap-treated astrocytes. Cultured astrocytes were loaded for 30 min with 50 µM $DCFH_2$-DA and then incubated for 5 min without or with 20 or 100 µM β-lap in the absence or the presence of 30 µM dicoumarol. Subsequently, the cells were lysed and the DCF fluorescence was quantified in the lysate supernatant. The data shown are means ± SD of relative values (normalised to the control: incubation in the absence of β-lap) obtained in 3 experiments performed on independently prepared cultures (n=3). Significant differences (ANOVA) between data obtained for incubations in the absence (0 µM) and the presence of β-lap are indicated by asterisks (*$p<0.05$, ***$p<0.001$).

Reversibility of β-lap induced GSSG accumulation

To test for the capacity of astrocytes to regenerate the normal high GSH to GSSG ratio after removal of β-lap and to investigate the importance of the availability of glucose for this process, the cells were deprived of glucose for 20 min and pre-incubated for 10 min with 20 µM β-lap in glucose-free IB to induce cellular GSSG accumulation. Subsequently, the β-lap was removed by washing and the cells were incubated for

1 min or 5 min without or with 5 mM glucose before the contents of cellular GSx and GSSG were determined. After the pre-incubation with β-lap the cellular GSx represented almost exclusively GSSG (Fig. 7) and a high GSSG to GSx ratio remained during a subsequent incubation in the absence of glucose (Fig. 7). In contrast, already after 1 min of incubation of β-lap-preincubated cells in the presence of glucose the cellular GSx contents (Fig. 7a) represented almost exclusively GSH as GSSG accounted to only 5 % (1 min) and 2 % (5 min) of the respective cellular GSx content (Fig. 7b).

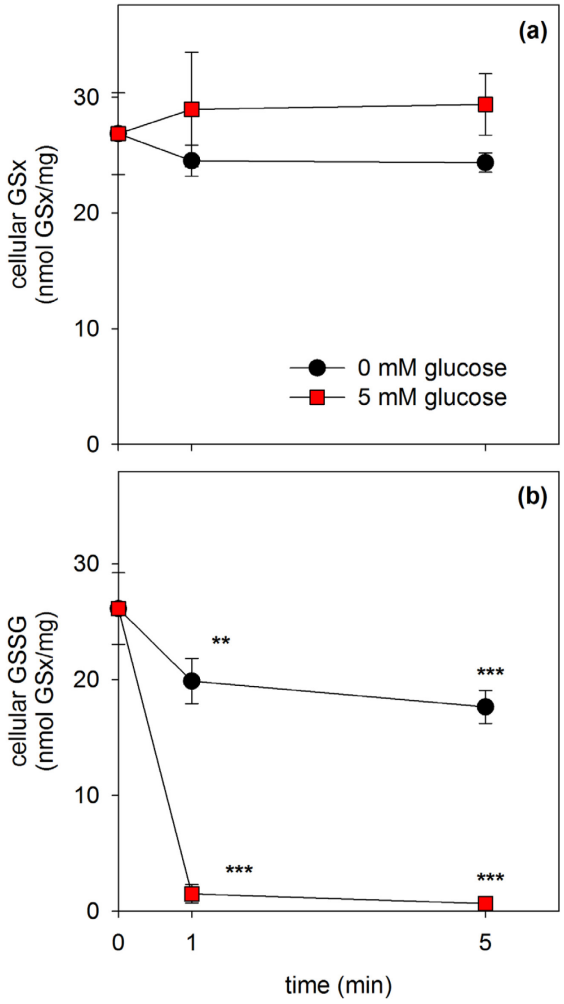

Figure 7: Reduction of the high β-lap-induced cellular GSSG levels after removal of β-lap. Astrocytes were preincubated for 20 min without glucose and subsequently for additional 10 min without glucose in the presence of 20 µM β-lap. After this preincubation, β-lap was removed by washing and the cells were incubated for 5 min in the absence or the presence of 5 mM glucose for up to 5 min before cellular GSx (a) and GSSG (b) contents were determined. The initial specific GSx and GSSG contents of the cultures were 42 ± 1 nmol/mg and 1 ± 0 nmol/mg, respectively. The protein content of the cultures was 132 ± 6 µg/well. The data shown are means ± SD of values obtained in 3 experiments performed on independently prepared cultures (n=3). Significant differences (ANOVA) compared to the data determined for the onset of the main incubation (t=0 min) are indicated by asterisks (**p<0.01, ***p<0.001).

Consequences of an application of dicoumarol and/or SOD plus catalase on the GSSG content in β-lap-treated astrocytes

To test whether application of dicoumarol is able to lower the high GSSG to GSx ratio found for β-lap-pretreated astrocytes, the cells were pre-incubated for 10 min with 20 µM β-lap which caused a high level of cellular GSSG (Fig. 8b). Application of a small volume of the solvent (IB), did not affect the high GSSG (Fig. 8b) to GSx (Fig. 8a, control) ratio during a subsequent incubation of up to 10 min. In contrast, application of dicoumarol to a final concentration of 30 µM lowered the high initial cellular GSSG content already by around 60 % within 1 min and after 10 min of incubation hardly any GSSG was detectable in the cells (Fig. 8b). In contrast, application of superoxide dismutase (SOD, EC 1.15.1.1) plus catalase (EC 1.11.1.6) had hardly any effect in lowering the cellular GSSG content of β-lap-exposed cells. However, application of dicoumarol plus the enzymes to astrocytes that were exposed to β-lap almost completely restored the high GSH to GSSG ratio of the treated astrocytes within 1 min of incubation to levels that are similar to those of untreated cells (Fig. 8b), suggesting that the applied enzymes remove extracellular ROS which are present in β-lap-treated astrocyte cultures and contribute to the maintenance of a high cellular GSSH to GSH ratio.

Test for the suitability of β-lap as electron cycler to mediate extracellular WST1 reduction

The NQO1 substrate menadione has been reported to serve for cultured astrocytes as membrane permeable electron cycler that enables the transfer of electrons from intracellular sources for extracellular reduction of the membrane-impermeable tetrazolium dye WST1 (Stapelfeldt *et al.* 2017). Therefore, also β-lap was considered as potential electron cycler that can shuttle electrons in its labile reduced form from intracellular sources to extracellular WST1. To test for this option, astrocytes were incubated with 20 µM β-lap and 400 µM WST1 in glucose-containing IB for up to 30 min before the extracellular concentration of WST1 formazan was photometrically determined. Indeed, the presence of β-lap allowed efficient WST1 reduction as demonstrated by the strong increase in extracellular WST1 formazan content (Fig. 9).

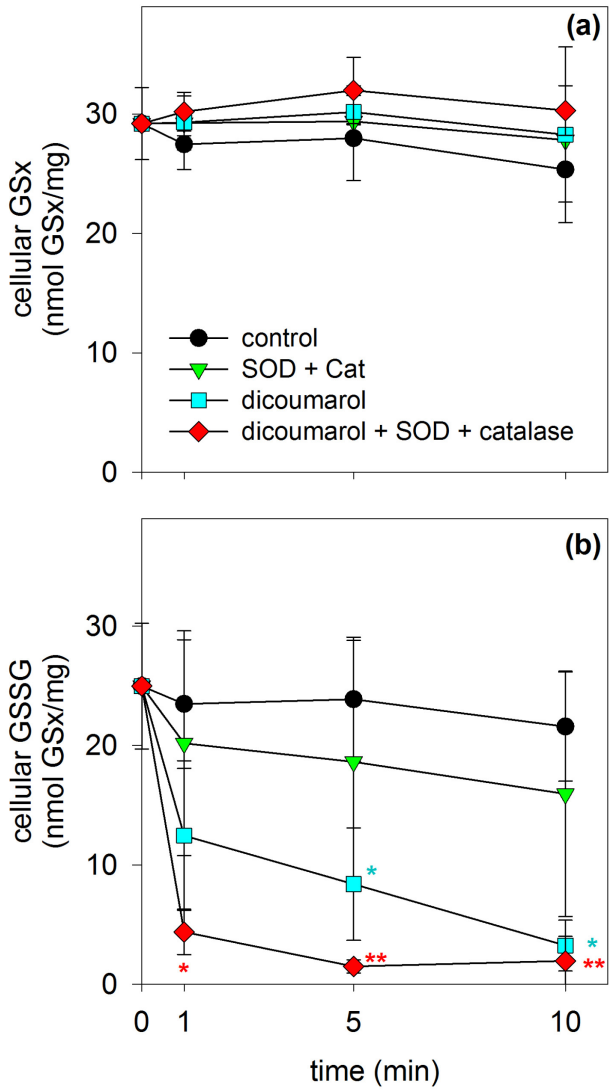

Figure 8: Consequences of an application of dicoumarol, SOD and catalase on the GSx and GSSG contents of β-lap-treated cultured astrocytes. Astrocytes were preincubated for 10 min with 20 µM β-lap before dicoumarol (final concentration of 30 µM) and/or SOD (100 U) plus catalase (260 U) were applied to the medium to start the main incubation of up to 10 min. For the indicated incubation periods the cellular GSx (a) and GSSG (b) contents were determined. The data shown are means ± SD of values obtained in 3 experiments performed on independently prepared cultures (n=3). The initial specific GSx content of the cultures was 40 ± 2 nmol/mg, the initial specific GSSG content was below the detection limit of the assay used. The protein content of the cultures was 127 ± 14 µg/well. Significant differences (ANOVA) compared to the data obtained for the control condition (application of solvent) are indicated by asterisks written in the colours of the respective symbols (*$p<0.05$, **$p<0.01$).

To investigate which β-lapachol-derived ROS may be involved in extracellular WST1 reduction, the detoxifying enzymes catalase and/or SOD were applied. The β-lap-dependent WST1 reduction was almost completely prevented in the presence of dicoumarol alone or of dicoumarol plus catalase plus SOD. In the absence of dicoumarol the presence of SOD lowered extracellular WST1 reduction by around 50 %, while catalase alone did not affect WST1 reduction and catalase in combination with SOD did not enhance the observed effect of SOD alone (Fig. 9).

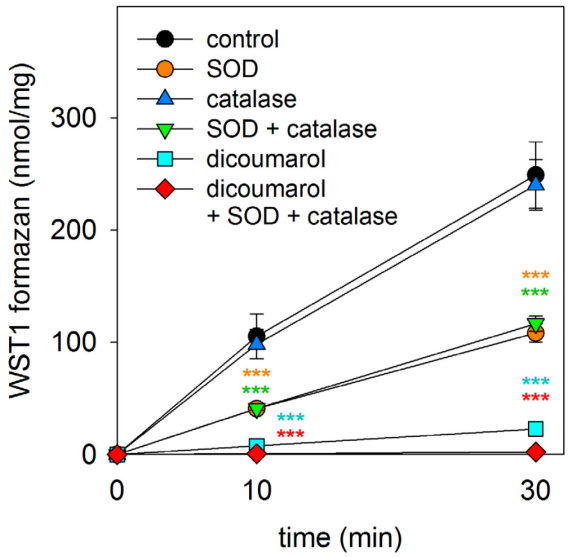

Figure 9: Use of β-lap as electron cycler to facilitate extracellular WST1 reduction by cultured astrocytes. The cells were incubated with 400 µM WST1 and 20 µM β-lap in the absence (control) or the presence of 30 µM dicoumarol, SOD (100 U) and/or catalase (260 U) for up to 30 min before the extracellular content of WST1 formazan was determined. The data shown are means ± SD of values obtained in 3 experiments performed on independently prepared cultures (n=3). The protein content of the cultures was 148 ± 23 µg/well. Significant differences (ANOVA) compared to the data obtained for the control conditions are indicated by asterisks written in the colours of the respective symbols (***$p<0.001$).

Discussion

Exposure of cancer cells to β-lap has been reported to induce toxicity and apoptosis (Don *et al.* 2001, Ough *et al.* 2005, Jeon *et al.* 2015, Bang *et al.* 2016, Xu *et al.* 2016, Silvers *et al.* 2017, Yang *et al.* 2017) which involves intracellular superoxide formation and oxidative stress (Bey *et al.* 2013) due to futile redox cycling by NQO1 (Siegel *et al.* 2012b, Bey *et al.* 2013, Silvers *et al.* 2017). As also astrocytes contain NQO1 (Park *et al.* 2016) the consequences of an exposure of astrocyte cultures to β-lap was investigated. Presence of β-lap in concentrations above 10 µM caused within minutes a rapid concentration-dependent ROS formation and GSH oxidation that was followed by a slower impairment of glycolytic lactate production and finally in compromised cell membrane integrity. NQO1 is able to use both NADH and NADPH as electron donor (Edwards *et al.* 1980). Thus, the slowed lactate production by astrocytes after application of higher concentrations of β-lap may be the consequence of a NQO1-dependent consumption of glycolytically generated NADH and of an accelerated metabolism of glucose-6-phosphate by the pentose phosphate pathway to compensate for the NQO1-dependent consumption of NADPH. Furthermore, lowered NAD^+ levels due to the activation of poly-(ADP-ribose) polymerase (PARP) during acute oxidative stress (Silvers *et al.* 2017) may also contribute to the lower glycolytic activity in β-lap treated astrocytes.

All the adverse β-lap-induced adverse effects on cultured astrocytes were completely prevented by the NQO1 inhibitor dicoumarol (Ernster *et al.* 1960, Hollander and Ernster 1975), consistent with the view that NQO1 plays the central role in generating β-lap-induced oxidative stress (Bey *et al.* 2013, Silvers *et al.* 2017). Concerning the potential application of β-lap as anti-tumor treatment (Li *et al.* 2014, Bang *et al.* 2016), it should be considered that also normal tissues cells which contain substantial NQO1 activity, such as brain astrocytes, may be affected by β-lap-induced oxidative stress.

Recently we have reported that the quinone and NQO1 substrate menadione induces oxidative stress and rapid GSH oxidation in cultured astrocytes (Steinmeier and Dringen 2019). However, the consequences of a menadione exposure of astrocytes cannot be prevented by application of the NQO1 inhibitor dicoumarol due to NQO1-independent ROS formation by menadione (Steinmeier and Dringen 2019). This strongly contrast to the results observed for β-lap-treated astrocytes, where ROS formation and GSSG accumulation were completely prevented by dicoumarol, suggesting that the oxidative stress observed in β-lap-treated astrocytes exclusively depends on the activity of NQO1. Thus, the sequential application of β-lap and

dicoumarol displays a suitable experimental system to rapidly induce (application of β-lap) and terminate (addition of dicoumarol) the duration of an acute oxidative stress condition, at least for cultured astrocytes. Such an experimental paradigm that clearly defines an experimental setting for studying consequences of oxidative stress might also by suitable for other types of cultured cells that contain substantial activities of NQO1. However, it should be considered that such reactions lead to an excessive consumption of reduced nicotinamide coenzymes, NADH and NADPH, which may affect metabolic and protective pathways during the treatment.

In cell lysates of astrocytes dicoumarol has been shown to inhibit NQO1 activity with half-maximal inhibition in the nM range (Ehrke *et al.* 2020).However, for intact astrocytes even at a concentration of 1 µM extracellular dicoumarol was unable to completely inhibit NQO1-dependent β-lap-mediated GSH oxidation, as evident by the small but significant increase in the GSSG to GSH ratio of astrocytes that had been exposed to β-lap plus dicoumarol for 5 min. Most likely higher extracellular concentrations of dicoumarol have to be applied to generate a sufficiently high intracellular concentration of dicoumarol to completely inhibit NQO1. This was achieved by the application of 30 µM dicoumarol which completely prevented NQO1-dependent β-lap-mediated ROS formation and GSSG accumulation. However, application of such high micromolar concentrations of dicoumarol has the disadvantage that also the export of GSH, GSSG and GSH-conjugates from astrocytes via the multidrug resistance protein 1 (Mrp1) is inhibited (Raabe *et al.* 2019).

The cellular consequences of an application of β-lap to cultured astrocytes that lead to ROS formation and GSSG oxidation are schematically shown in figure 9. Reduction of β-lap by NQO1 generates the instable β-lapachol which generates two molecules of superoxide during its auto-oxidation to β-lap (Pink *et al.* 2000, Silvers *et al.* 2017). In cells, superoxide is rapidly disproportionated by SODs to oxygen and H_2O_2 (Canada and Calabrese 1989) and the peroxide can be reduced to water by GPx which takes the electrons required from GSH and generates GSSG (Dringen *et al.* 2015) . Cellular GSSG is reduced to GSH in the reaction catalysed by GR which uses NADPH as electron source (Dringen and Gutterer 2002). For β-lap-treated astrocytes a strong accumulation of GSSG was observed, indicating that for those conditions the rate of GSH oxidation by GPx is strongly exceeding the rate of GR-mediated GSSG reduction as previously reported for astrocytes that had been exposed to acute or chronic H_2O_2-stress (Dringen and Hamprecht 1997, Hirrlinger *et al.* 2002a). One consequence of the strong accumulation of GSSG in β-lap-treated astrocytes is the export of GSSG, which is mediated by the multidrug resistance protein 1 (Mrp1) (Hirrlinger *et al.* 2001,

Minich *et al.* 2006) and has previously been reported for conditions that induce severe oxidative stress in cultured astrocytes (Hirrlinger *et al.* 2001, Minich *et al.* 2006, Raabe *et al.* 2019, Steinmeier and Dringen 2019).

Upon termination of the β-lap-induced oxidative stress, either by removal of β-lap or by inhibiting NQO1-mediated β-lap reduction by application of dicoumarol, the level of cellular GSSG declined rapidly and the normal high ratio of GSH to GSSG was re-established within minutes, demonstrating the high capacity of astrocytes to efficiently reduce GSSG by the GR reaction. The rapid GSSG reduction was strongly impaired in glucose-deprived astrocytes, confirming the reported importance of the glucose metabolism via the pentose phosphate pathways (PPP) for providing the NADPH required for GR-dependent GSSG reduction (Kussmaul *et al.* 1999, Dringen *et al.* 2015). The rapid regeneration of the high GSH to GSSG ratio in glucose-fed astrocytes demonstrates also that an irreversible inhibition of GR or of the NADPH-regeneration by the PPP which also would lead to a strong GSSG accumulation in β-lap-treated astrocytes can be excluded.

Although a rapid regeneration of cellular GSSG was observed after application of dicoumarol to the β-lap-containing incubation medium, this dicoumarol-initiated cellular GSSG reduction was accelerated by co-application of dicoumarol with SOD plus catalase. As these enzymes cannot penetrate an intact cell membrane, it was concluded that release of ROS and/or extracellular generation of ROS contribute to the oxidative stress generated after application of β-lap (Fig. 10). As shown for menadione (Stapelfeldt *et al.* 2017), also the β-lap/β-lapachol redox pair was found to efficiently act as electron cyclers and to mediate the electron transfer from cellular sources for extracellular WST1 reduction which requires export of β-lapachol and reuptake of β-lap (Fig. 10). As H_2O_2 does not chemically reduce WST1 (data not shown) and as the presence of SOD, but not of catalase, lowered the extracellular WST1-reduction in presence of β-lap by at least 50%, it was concluded that β-lapachol had been indeed released from β-lap-treated astrocytes and that this labile compound generated extracellularly superoxide which was used for WST1 reduction. However, as superoxide can be released from cells (Meier *et al.* 1989), we can currently also not exclude that intracellular β-lapachol-derived superoxide may be released from astrocytes and contribute to the observed extracellular WST1 reduction. In addition, extracellular H_2O_2 originating from cellular or extracellular β-lapachol-derived superoxide could have contributed to the slow regeneration of the normal high cellular GSH to GSSG ratio after uptake into the cells and subsequent cellular clearance via GSH and GPx after termination of the oxidative stress by application of dicoumarol.

Thus, for efficient and immediate termination of β-lap-induced oxidative stress it is recommended to apply both dicoumarol and SOD plus catalase in order to inhibit the formation of new ROS and to eliminate the extracellular reservoir of ROS, respectively.

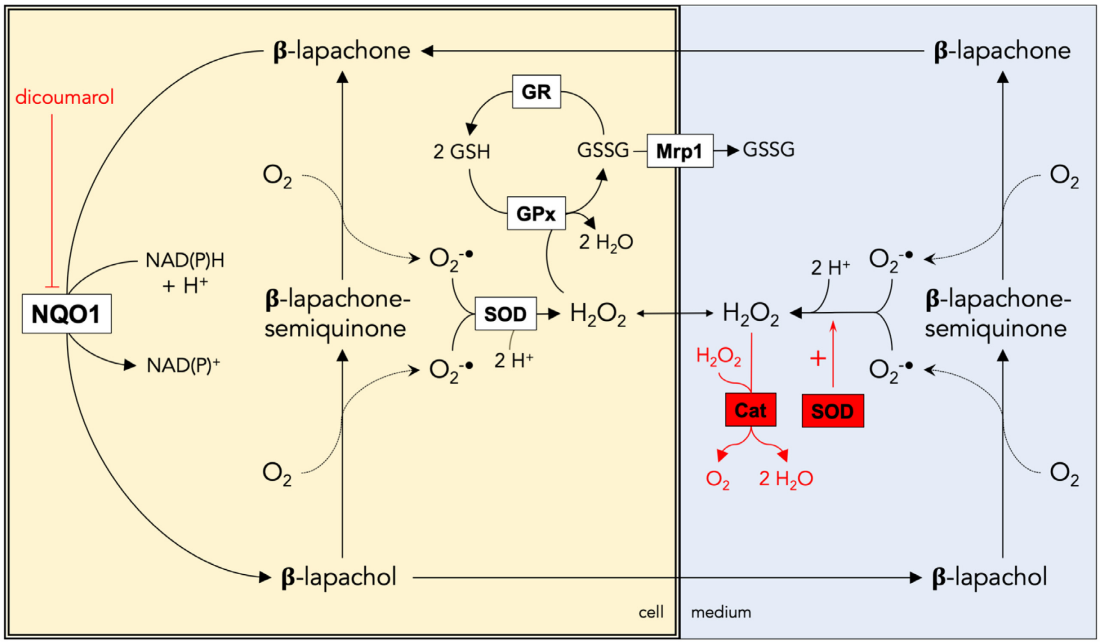

Figure 10: Consequences of a treatment of astrocytes with β-lap. β-lap is reduced within astrocytes by the dicoumarol-sensitive NQO1 in a two-electron transfer reaction to β-lapachol. The labile β-lapachol can auto-oxidise inside of the cell in two distinct oxidation steps first to β-lapachone-semiquinone and then to β-lapachone, thereby producing 2 molecules of superoxide. Superoxide is rapidly disproportionated by superoxide dismutase (SOD) to oxygen and H_2O_2. Cellular reduction of H_2O_2 by glutathione peroxidase (GPx) leads to the oxidation of GSH and the formation of cellular GSSG which can subsequently be reduced to GSH by the NADPH-dependent glutathione reductase (GR) or be released from the cells via multidrug resistance protein 1 (Mrp1). β-lapachol is membrane-permeable and can be released from astrocytes. Auto oxidation of extracellular β-lapachol will generate extracellular superoxide which can either mediate WST1 reduction or can chemically disproportionate to H_2O_2.This H_2O_2 can enter the astrocytes and can be detoxified by the astrocytic GPx. Application of the NQO1 inhibitor dicoumarol, prevents all effects observed for a treatment of astrocytes with β-lap, demonstrating the central function of NQO1 in generating the β-lap-induced oxidative stress. Application of SOD and catalase (Cat) to β-lap-treated cells rapidly removes extracellular superoxide and H_2O_2, thereby preventing cellular GSH oxidation caused by GPx-mediated reduction of extracellular H_2O_2 that was taken up by the cells.

Unexpectedly, the extracellular lactate release in presence of β-lap and 30 µM dicoumarol was found elevated compared to the values obtained for control cells. Previous work from our group suggests that in cultured astrocytes NADPH may be the preferred electron donor to deliver electrons for NQO1-dependent reactions (Ehrke *et al.* 2020). Assuming that the consumption of NADPH is lowered by dicoumarol-mediated inhibition of cytosolic NQO1, less glucose-6-phosphate would be

needed as substrate for NADPH regeneration by the pentose phosphate cycle (Dringen *et al.* 2007) and more glucose-6-phosphate could be used for glycolytic lactate production. However, we can also not exclude a potential direct action of dicoumarol on mitochondrial processes which may lower mitochondrial ATP production and as a consequence increase glycolytic flux as previously shown for several compounds (Hohnholt *et al.* 2017, Arend *et al.* 2019).

In conclusion, application of micromolar concentrations of β-lap induces severe oxidative stress in cultured astrocytes, as evident by accelerated ROS production and strong GSSG accumulation. Such severe consequences are important to be considered for potential systemic application of β-lap as anti-tumor drug. Although data on the permeability of β-lap through the blood-brain barrier are missing so far (Lee *et al.* 2018), this compound is due to its hydrophobicity (Bermejo *et al.* 2017) quite likely to cross this barrier after systemic application and thereby is likely to interact with astrocytes in brain. As the β-lap-induced oxidative stress formation in cultured astrocytes depends exclusively on the activity of the enzyme NQO1 which can be efficiently inactivated by dicoumarol, the sequential application of β-lap and dicoumarol is a valuable experimental setup to rapidly induce and terminate an acute oxidative stress condition in NQO1 expressing cultured cells. This experimental setting could be useful for studying export of GSSG during oxidative stress, the cellular mechanisms required to regenerate GSH or the metabolic processes that provide the NADPH required for GSSG reduction after terminating the oxidative stress. With some adaptations, the experimental setting should also be applicable for studies of the consequences of oxidative stress in other types of cultured cells as long as these cells display sufficient activity of NQO1.

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3. Summarizing Discussion

3.1 Participation of NAD(P)H: quinone acceptor oxidoreductase 1 in the menadione and beta-lapachone-mediated reduction of the water-soluble tetrazolium salt 1 in astrocytes

This thesis shows that the adverse consequences on astrocytes mediated by menadione or beta-lapachone are very similar but do not exclusively rely on the activity of the antioxidative enzyme NQO1. Regarding beta-lapachone, all observed adverse effects on astrocytes could be prevented by the NQO1-inhibitor dicoumarol and therefore NQO1 is believed to be the solely responsible enzyme in the harmful activation of beta-lapachone in astrocytes, leading to the formation of ROS, GSSG and loss in cellular viability (all described in further detail in chapters 3.2 and 3.3). In contrast, even though an incubation of astrocytes with menadione lead to similar effects as compared to beta-lapachone, the menadione-dependent formation of GSSG in astrocytes could not be blocked by dicoumarol, leading to the assumption that most of the menadione-dependent consequences on astrocytes (all described in further detail in chapters 3.2 and 3.3) do not rely on the activity of NQO1, but are either due to a chemical mediation by menadione or an dicoumarol-insensitive enzyme.

The presence of NQO1 in rat astrocyte primary cultures was proved by immunochemical detection of NQO1 in astrocytes and by quantification of the enzymatic activity of NQO1 (chapter 2.1). As NQO1 is known to reduce the quinone compounds menadione (Lind *et al.* 1990, Rooseboom *et al.* 2004) and beta-lapachone (Chakrabarti *et al.* 2015, Bang *et al.* 2016, Huang *et al.* 2016, Silvers *et al.* 2017) to menadiol and beta-lapachol, respectively, NQO1 is the most likely enzyme for two-electron reduction of the two quinone compounds.

NQO1 is suggested as the central enzyme in the menadione-mediated WST1 assay (Ehrke *et al.* 2020). This assay was originally designed on intact astrocyte primary cultures for the quantification of metabolic activity of cultured neural cells (Stapelfeldt *et al.* 2017). In the WST1 assay, menadione is used as electron cycler to shuttle electrons from intracellular sources to the membrane impermeable and hence extracellular WST1, allowing for its reduction to the detectable WST1 formazan (figure 1.3 in chapter 1.2.1). The essential participation of NQO1 in this menadione-dependent process is confirmed by the complete inhibition of WST1 reduction upon addition of 1 µM dicoumarol and the determined very low and therefore reasonably selective K_{ic} values of dicoumarol towards NQO1 of 1.2 nM for NADH and 5.9 nM for

NADPH (Ehrke *et al.* 2020) that are similar to literature data (Hosoda *et al.* 1974). Also, the unusual characteristic of NQO1 to use both nicotinamide nucleotide cofactors NADH and NADPH with roughly equal efficiency (Ernster *et al.* 1962, Ernster *et al.* 1972) for the reduction of WST1, as also shown for lysates prepared from astrocyte cultures (Ehrke *et al.* 2020) confirms the participation of NQO1 in the menadione-mediated WST1 reduction. Additionally, the mainly cytosolic (Ernster *et al.* 1972, Edwards *et al.* 1980, Dinkova-Kostova and Talalay 2010, Siegel *et al.* 2012a) and only minimal mitochondrial (Lind *et al.* 1990) or membrane-associated (Lind *et al.* 1990) localisation of NQO1 enzymatic activity described in literature is consistent with the localisation studies performed for the menadione-mediated WST1 reduction capacity of astrocytes (Ehrke *et al.* 2020).

In analogy to menadione, beta-lapachone was also tested for its ability to substitute menadione and act as electron cycler in the WST1 assay system. It was shown that also presence of beta-lapachone lead to robust formation of WST1 formazan in astrocyte cultures and that the reduction of WST1 using beta-lapachone as electron cycler is also inhibited by dicoumarol (figure 9 in chapter 2.3) as previously shown for menadione (Ehrke *et al.* 2020). As suggested previously (Chakrabarti *et al.* 2015, Huang *et al.* 2016, Beg *et al.* 2017, Silvers *et al.* 2017), it is therefore reasonable to assume that also in rat astrocyte cultures NQO1 is the responsible enzyme for the reduction of beta-lapachone. A replacement of menadione by beta-lapachone in the metabolic WST1 assay (Stapelfeldt *et al.* 2017) as electron cycler is therefore potentially possible. In the original WST1 assay, the menadione concentration used is 50 µM (Stapelfeldt *et al.* 2017), that has been proven to e.g. induce heavy formation of GSSG in astrocytes and a severe decrease in the lactate released from astrocyte cultures into the medium (chapter 2.2). A concentration of only 10 µM beta-lapachone has been shown to lead to promising results in preliminary experiments (data not shown), as this concentration of beta-lapachone does only induce little GSSG accumulation and no alteration in lactate release of astrocytes (chapter 2.3), while simultaneously providing suitable WST1 formazan reduction (data not shown). As it is desirable to decrease side effects of the WST1 assay system itself, menadione should consequently be substituted by beta-lapachone.

The well characterized and best described NQO1 inhibitor dicoumarol does however also show off-target effects (Timson 2017, Pey *et al.* 2019), including the formation of mitochondria-derived superoxide production (Gonzalez-Aragon *et al.* 2007), the inhibition of UDP-glucuronosyltransferase (Segura-Aguilar *et al.* 1986) or inhibition of Mrp1-mediated GSSG export from astrocytes (Raabe *et al.* 2019). It is therefore

recommended to conduct further studies on the participation of NQO1 in the reduction of menadione and beta-lapachone in astrocyte cultures using different inhibitors of NQO1 distinct from dicoumarol that show less off-target effects, e.g. coumarin-based compounds as described by Scott *et al.* (2011) or the indolequinone ES936, a described suicide inhibitor of NQO1 (Dehn *et al.* 2006).

3.2 Effects of menadione and beta-lapachone on the glutathione metabolism of astrocytes

This thesis shows that exposure of astrocytes to the quinone-compounds menadione or beta-lapachone results in the formation of ROS and the rapid and strong accumulation of cellular GSSG. Especially menadione lead to severe losses of the total GSx content of astrocytes, while beta-lapachone caused comparably less GSx depletion in astrocytes. The menadione-dependent consequences on astrocytes are not inhibited by the NQO1 inhibitor dicoumarol, while all described adverse consequences of beta-lapachone were prevented. Therefore, it is assumed that menadione-mediated GSH oxidation and GSx depletion are not primarily due to catalysis by NQO1 but are due to a dicoumarol-insensitive enzyme or chemical reactions of menadione within cells. Simultaneously, for beta-lapachone a mostly NQO1-dependent bioactivation in rat astrocyte cultures to its unstable hydroquinone is assumed that leads to formation of GSSG and toxicity in astrocytes. Furthermore, both menadione and beta-lapachone induced intracellular GSSG was only efficiently recycled to GSH in a glucose-dependent manner and specifically for menadione also the protective influence of glucose on the formation of GSSG was described. The adverse consequences observed for a treatment of astrocytes with menadione or beta-lapachone are summarized in table 3.1.

To further illustrate differences in menadione and beta-lapachone-induced GSSG formation and loss of total GSx, dose-response curves obtained for the tow compounds were compared (figure 3.1). For the initial increase of total GSSG upon exposure of astrocytes to menadione or beta-lapachone for 5 min, EC_{50} values of 68 $\pm$ 34 µM and 13 $\pm$ 1 µM, respectively, were determined (figure 3.1a). This pointed towards a lower half maximal effective concentration of beta-lapachone in comparison to menadione, even though the menadione concentrations investigated for the 5 min timepoint were not ideal for EC_{50} determination. Exemplary, 20 µM of beta-lapachone lead to a more pronounced shift in the GSH/GSSG ratio than the highest menadione concentration investigated here, reaching as much as 84 % of the total GSx being GSSG within 5 min. In comparison, induced GSSG formation by 100 µM menadione only reached as much as 64 % of total GSx being GSSG within 5 min of exposure (figure 3.1a).

Table 3.1: Adverse consequences of menadione and beta-lapachone on astrocyte-rich primary cultures.

Effect	Menadione	Beta-lapachone
Glutathione metabolism		
total GSSG (5 min)		
formation as % of total GSx (most effective concentration)	64 % (100 µM)	94 % (100 µM)
EC_{50} value	68 ± 34 µM	13 ± 1 µM
total GSx (2 h)		
% of initial GSx (most effective concentration)	35 % (200 µM)	72 % (100 µM)
EC_{50} value	66 ± 13 µM	18 ± 1 µM
glucose-dependent		
recycling of GSSG	yes	yes
formation of cellular ROS	yes	n.d.
attenuation of GSSG formation	yes	n.d.
Confirmation of ROS generation		
method used	R123 formation	DCF fluorescence
superoxide detection	n.d.	WST1 assay with SOD/Cat
Vitality		
significant release of cellular LDH (most effective concentration)	4 h (100 µM)	30 min (20 µM)
significant decrease in lactate release (most effective concentration)	1 h (100 µM)	30 min (30 µM)
Dicoumarol		
prevents adverse consequences	no	yes

Data for menadione are from chapter 2.2 and data for beta-lapachone are from chapter 2.3 of this thesis. EC_{50} values were determined using the data described in the respective chapters. R123: rhodamine123 staining; DCF: dichlorofluorescein; EC_{50}: half-maximal effective concentration, LDH: lactate dehydrogenase; SOD: superoxide dismutase; Cat: catalase; WST1: water soluble tetrazolium salt 1; n.d.: not determined.

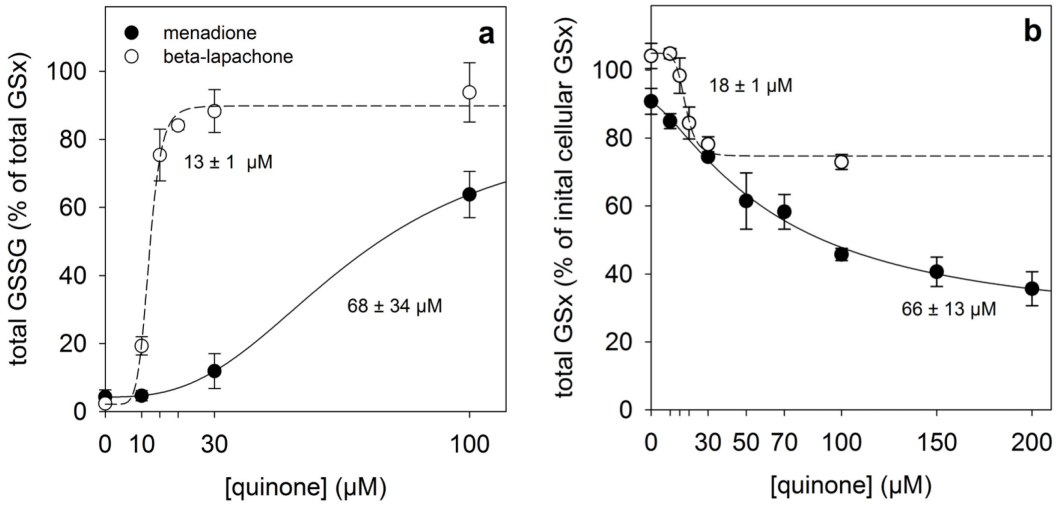


Figure 3.1: Dose-response curves of beta-lapachone and menadione on the GSSG and GSx content of astrocytes. Dose-response curves were prepared from data in chapters 2.2 and 2.3 and depict menadione and beta-lapachone concentration-dependent formation of total GSSG within 5 min (a) and the loss in total GSx within 2 h (b). EC_{50}-values are given as mean ± SD.

Analysis of the loss of total GSx after 2 h of exposure of astrocytes to menadione or beta-lapachone revealed EC_{50} values of 66 ± 13 µM and 18 ± 1 µM, respectively (figure 3.1b). Albeit the EC_{50} value for beta-lapachone was determined to be lower, the maximal loss in total GSx was more pronounced in menadione-treated astrocytes, leading to a loss of total GSx to 35 % of the initial GSx as opposed to only to 72 % in presence of beta-lapachone. Notably, the calculated EC_{50} values for the beta-lapachone-mediated adverse consequences on astrocytes described were also in the range of half maximal lethal doses reported for beta lapachone in cancer cell lines expressing NQO1, ranging between 1 and 20 µM of beta-lapachone within 2 h (Huang *et al.* 2016).

Menadione has been used to induce GSSG accumulation in astrocytes and to study the export of GSSG by Mrp1 (Raabe *et al.* 2019). However, beta-lapachone is more effective in rapidly inducing GSSG accumulation in astrocytes (Fig. 3.1a) and leads to less depletion of total GSx as compared to menadione (Fig. 3.1b). This suggests beta-lapachone as a good alternative to menadione to study the consequences of high cellular GSSG levels in astrocytes, with less side effects to be expected with respect to menadione.

For beta-lapachone it has been shown that bioactivation by NQO1 is responsible for the formation of ROS and ultimately cell death (Bey *et al.* 2013, Chakrabarti *et al.* 2015, Silvers *et al.* 2017). Although the two-electron reduction of

NQO1 is preventing the direct formation of a semiquinone-radical that can be beneficial for the cell by lowering the burden of oxidative stress (Dinkova-Kostova and Talalay 2010, Siegel *et al.* 2012a, Pey *et al.* 2019), in the case of beta-lapachone reduction by NQO1 leads to formation of the unstable hydroquinone beta-lapachol that easily takes part in redox cycling (Pink *et al.* 2000, Kung *et al.* 2014). This is the likely reason why treatment of astrocytes with beta-lapachone leads to superoxide production and ROS formation (chapter 2.3). After auto-oxidation back to the parent quinone form, beta-lapachone is then again available to start a new cycle of reduction and auto-oxidation. This beta-lapachone induced futile redox cycling and ROS production is the most likely reason for the extensive GSH oxidation in beta-lapachone-treated astrocytes, as GSH reacts chemically and by enzymatic catalysis with ROS (Dringen *et al.* 2015) and a comparable cellular accumulation of GSSG upon treatment of astrocytes with H_2O_2 has previously been shown (Liddell *et al.* 2009).

The information available on beta-lapachone used on astrocytes is scarce and also little is known about its effect on the glutathione metabolism. A study by Park *et al.* (2016) reported a steady increase of total GSx upon treatment of rat primary cultures with a beta-lapachone concentration as low as 1 µM, associated with an upregulation of other enzymes and proteins under the control of the Nrf2/Keap1/ARE-pathway. However, this increased GSx content seemed to be the result of an accelerated glutathione synthesis (Park *et al.* 2016). Also, exposure of breast cancer cells to beta-lapachone has been reported to lead to light GSSG formation (roughly 30 % of total GSx), albeit in the low hour range (Bey *et al.* 2013) and not within 5 min as reported here.

For menadione an NQO1-independent mechanism for the extensive GSSG formation in rat astrocyte cultures is likely (chapter 2.2). However, the occurrence of GSSG after menadione-treatment of cells has been reported (Peuchen *et al.* 1996, Klaus *et al.* 2010), as well as a depletion in GSH of astrocytes upon treatment with menadione (Rodrigues *et al.* 2017).

The quinone menadione can be reduced by other cellular reductases, e.g. cytochrome P450 reductases (Floreani and Carpenedo 1992, Bayol-Denizot *et al.* 2000, Rooseboom *et al.* 2004). Formation of the produced menadione semiquinone-radical is directly connected to superoxide formation in the presence of molecular oxygen, followed by formation of H_2O_2 and a cellular detoxification by GSH resulting in GSSG (Klotz *et al.* 2014). In literature, the formation of superoxide after exposure of rat astrocytes to menadione has been shown already over two decades ago (Abe and Saito 1996, Bayol-Denizot *et al.* 2000).

Also, the oxidation and alkylation of numerous biomolecules is reported for menadione (Rooseboom *et al.* 2004, Klotz *et al.* 2014) and also the chemical ROS formation of menadione is described (Ross *et al.* 1985, Buffinton *et al.* 1989, Klotz *et al.* 2014). Menadione (2-methyl-1,4-naphtoquinone) can also chemically react with glutathione, forming the hydroquinone-conjugate 3-glutathionyl-2-methyl-1,4-naphtoquinol that (1) auto-oxidizes faster than the unsubstituted menadione and (2) is leading to GSx depletion due to GSH conjugation (Ross *et al.* 1985, Buffinton *et al.* 1989).

The conjugation of menadione with nucleophiles like GSH (Ross *et al.* 1985) might well be the reason for the severe observed loss in total GSx described in chapter 2.2. Herein, the available C3 position for conjugation of menadione and the sufficient electrophilicity due to the methyl-group in the C2 position seem to be an important factor (Klotz *et al.* 2014). Substitution of this methyl-group in the C2 position of menadione with a hydroxyl-group yields a naphthoquinone termed lawsone (figure 3.2). To test for the potential of lawsone to induce the same GSH oxidation and losses in viability as described for menadione in chapter 2.2, astrocyte cultures were incubated with 100 µM lawsone or menadione. The incubation of astrocytes with lawsone did not lead to any GSSG formation after 5 min nor to a loss in the total GSx or an increase in extracellular LDH activity after 4 h (figure 3.2) as previously described for menadione (chapter 2.2). The likely reason for this is that in lawsone, the electrophilicity of the C3 is decreased due to the hydroxyl-group that is pulling electrons, making lawsone less likely to conjugate with nucleophiles (Klaus *et al.* 2010, Klotz *et al.* 2014). Also, nearly no superoxide was detected upon incubation of keratinocytes with lawsone (Klaus *et al.* 2010). This is supporting the notion that chemical reactivity of menadione is the central reason for the observed rapid GSH oxidation and depletion of total GSx in astrocytes.

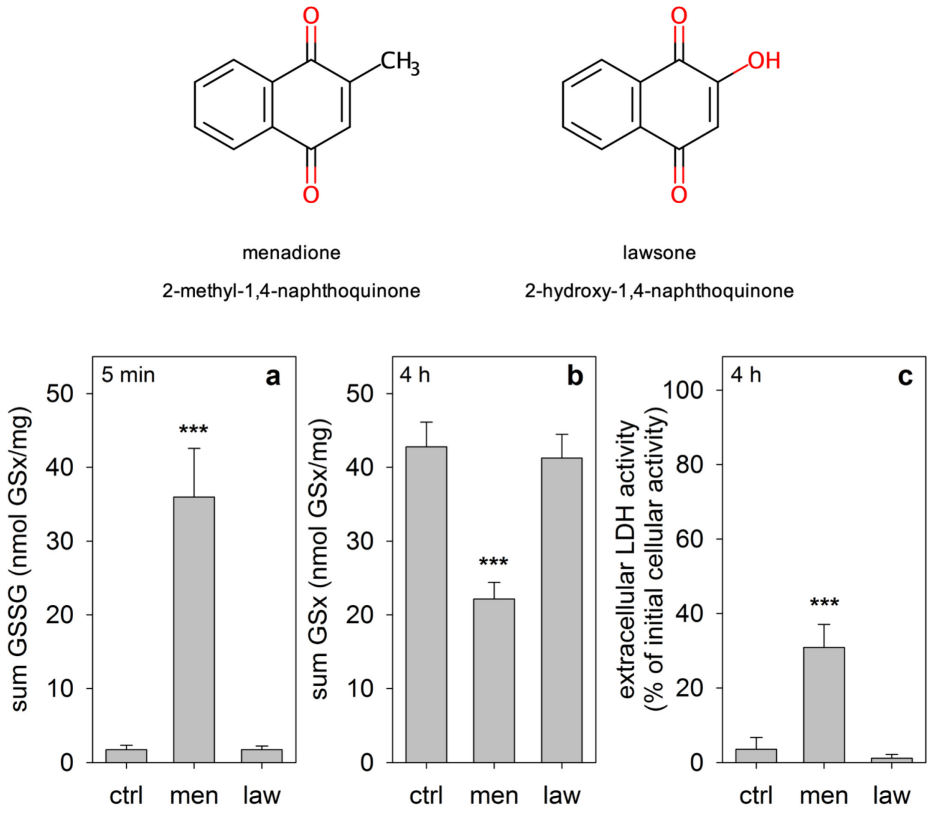


Figure 3.2: Chemical structures of menadione and lawsone and the effects of a treatment of astrocytes with either quinone compounds. Astrocytes were incubated without or with menadione or lawsone (chemical structures shown) in concentrations of 100 µM for the indicated time points as descried in chapter 2.2. Then contents of GSSG (a, 5 min) and GSx (b, 4 h) were determined as well as the activity of extracellular lactate dehydrogenase (LDH, c, 4 h). The data shown represent means ± SD of values obtained from three independently prepared cultures. The initial cellular GSx content was 49 ± 4 nmol/mg, initial cellular GSSG content was 1 ± 0 nmol GSx/mg and initial protein accounted for 121 ± 15 µg/well. The significance of differences compared to values obtained without quinone treatment (control) was analysed by ANOVA and is indicated by asterisks (***$p<0.001$). ctrl: control; men: menadione; law: lawsone.

3.3 Modulation of metabolism and membrane integrity of astrocytes by menadione and beta-lapachone

The results shown in this thesis provide also insights into the toxicity mediated by menadione and beta-lapachone on astrocyte cultures. Treatment of astrocytes with menadione or beta-lapachol caused substantial decreases of the lactate released from astrocytes and impaired the membrane integrity.

For cells in culture, the induction of toxicity by menadione or beta-lapachone has been described (Abe and Saito 1996, Bayol-Denizot *et al.* 2000, Pink *et al.* 2000, Klaus *et al.* 2010, Loor *et al.* 2010, Chakrabarti *et al.* 2015, Park *et al.* 2016, Beg *et al.* 2017, Rodrigues *et al.* 2017, Silvers *et al.* 2017). The likely reason for this is the increased level of ROS within cells exposed to these naphthoquinones. Especially for beta-lapachone it has been proven that within a window of 30 to 60 min of an exposure of cancer cell lines to beta-lapachone, cell necrosis was initiated (Silvers *et al.* 2017). This is similar to the impairment of membrane integrity as confirmed by the release of LDH from astrocytes following a beta-lapachone treatment (chapter 2.3). The initiation of necrosis was described to be due to a cascade started by the increases in cellular superoxide production and the following formation of ROS that damage DNA molecules, lipids and proteins as well as lead to Ca^{2+} release from the endoplasmic reticulum (Silvers *et al.* 2017). Consequently, the enzyme poly-(ADP-ribose) polymerase 1 (PARP1) that is responsible for the stabilisation and repair of DNA strand breaks becomes hyperactivated in the presence of Ca^{2+}. This PARP1 hyperactivation results in dramatic losses of NAD^+ and ATP, causing the cell to die (Bey *et al.* 2007, Silvers *et al.* 2017). Also, the damage of DNA has been described for the incubation of cells with menadione (Bayol-Denizot *et al.* 2000, Loor *et al.* 2010, Rodrigues *et al.* 2017) as well as beta-lapachone, including the evidence of severe losses in NAD^+ and also ATP (Chakrabarti *et al.* 2015, Huang *et al.* 2016) suggesting an impairment of membrane integrity in astrocytes also due to the redox-cycling of menadione or beta-lapachone.

The severe and fast drop in lactate released from astrocytes described in this thesis is very similar between a treatment of astrocytes with menadione and beta-lapachone, disregarding slight variations in the exact incubation times and substance concentrations. The glycolytic lactate production of astrocytes has been described to decrease upon exposure of astrocyte cultures to chronic H_2O_2 stress (Liddell *et al.* 2009) and has also been reported for pancreatic cancer cells exposed to beta-lapachone (Silvers *et al.* 2017). Likely, this is also the consequence of the rapid ROS formation and GSSG accumulation upon treatment of astrocytes with beta-

lapachone or menadione. The shift of the GSH/GSSG ratio is a trigger for the redirection of glucose from glycolysis into the pentose phosphate pathway to account for antioxidative defense and facilitate recycling of essential NADPH (Dringen *et al.* 2007, Brandes *et al.* 2009, Dringen *et al.* 2015, Mullarky and Cantley 2015). Moreover, the NQO1-dependent reduction of beta-lapachone is additionally using up NADH (Silvers *et al.* 2017) and losses in cellular NADH have also been reported for astrocytes treated with menadione (Ehrke *et al.* 2020). As NADH is needed by LDH to oxidize pyruvate to lactate the decreases of cellular NADH upon treatment of astrocytes with beta-lapachone or menadione may contribute to the decrease of lactate released from astrocytes. However, it is recommended to further elucidate the exact mechanisms underlying the described drop in lactate release in astrocytes upon quinone treatment.

In summary, the data shown in this thesis reveal severe GSH oxidation and losses in vitality of rat astrocyte cultures upon exposure to menadione or beta-lapachone. Experiments about the extent of blood-brain barrier penetration have not been performed for beta-lapachone so far (Lee *et al.* 2018). For menadione it is known that it is partly metabolized to menaquinone in mammals (Shearer *et al.* 2012) and that it is readily excreted in the urine (Thijssen *et al.* 2006), which would lower the active menadione concentrations after uptake. However, as both menadione (Thijssen *et al.* 2006) and beta-lapachone (Bermejo *et al.* 2017) are small lipophilic compounds it is expected that they readily cross the blood-brain barrier if circulating in the blood system. Therefore, brain cells are potentially encountering menadione or beta-lapachone upon treatment of patients with these compounds. Under the acceptance of rat primary astrocyte cultures as a model system for brain astrocytes, severe damages of astrocytes *in vivo* for a systemic application are to be expected if sufficiently high concentrations of menadione or beta-lapachone are reached in the brain. Consequently, it is highly recommended to elucidate side effects of menadione and beta-lapachone *in vivo* when considering the use of any of the two compounds as anti-cancer drugs in patients.

3.4 Future Perspectives

In the present thesis it was demonstrated that NQO1 takes a central part in the bioactivation of beta-lapachone in astrocytes and also the argumentation for an NQO1-independet mechanism regarding the GSH oxidation and loss in vitality in astrocyte cultures treated with menadione was supported. Therefore, it would be suitable to proof the role of NQO1 in the future by specifically turn off NQO1-mediated mechanisms in astrocyte primary cultures. A potent and frequently used technique for this is the downregulation of a specific protein on the mRNA level, using small interfering RNAs (siRNA) that induce RNA interference (RNAi) and cause a decrease in the expression of the respective protein (Hu *et al.* 2019). Astrocyte cultures pre-treated with siRNA specific for NQO1 should contain less or ideally no active NQO1, allowing for an investigation of the cultures in the absence of NQO1 enzymatic activity. This could provide further insights into the menadione- and beta-lapachone-dependent oxidation of GSH described in this thesis and could confirm the role of NQO1 in reduction of beta-lapachone in astrocyte primary cultures. Regarding the results described in this thesis for beta-lapachone, a prevention of GSH oxidation and of the loss in vitality in astrocyte cultures would be expected, and the persistence of some herein NQO1-attributed consequences would suggest the involvement of different mechanisms. For menadione, no severe changes in adverse consequences on astrocyte cultures would be expected, as the menadione-induced GSH oxidation and toxicity in astrocyte cultures seem to be NQO1-independent. In fact, initial experiments were performed to knock-down NQO1 using siNQO1 RNA in combination with lipofectamine in our astrocyte-rich primary cultures. However, the best result obtained in such studies so far was a lowering to approximately 60 % of NQO1 activity with respect to the control siRNA (data not shown) and further improvements are needed.

As transfection procedures are less effective in primary cultures (Karra and Dahm 2010, Alabdullah *et al.* 2019), immortalized cell lines could be used as an alternative in the future to knock down NQO1 and study the consequences of a treatment of menadione or beta-lapachone on astrocyte-like cells. Therefore, e.g. the glial tumor-derived immortalized cell line C6 could be used which is also used as a model system for astrocytes (Benda *et al.* 1968, Ozawa *et al.* 2019). Alternatively, the efficiency of transfection could be improved by using a virus-based transfection method (like adenovirus) that allow for highly efficient gene delivery *in vivo* and *in vitro* (Karra and Dahm 2010).

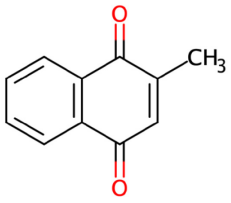
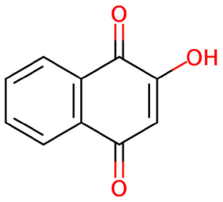
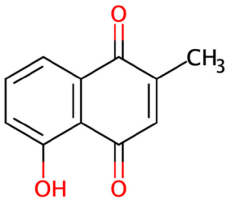
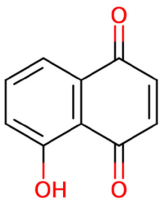
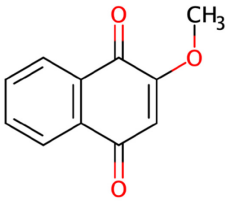
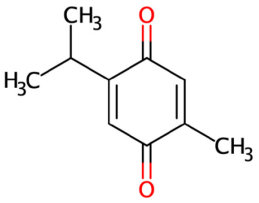
To completely avoid the problem of transfection of primary cultures to knock-down NQO1, the use of knock-out mice that do not express NQO1 at all is a suitable

perspective. This would either allow for the preparation of astrocyte cultures from mouse brain that do not contain NQO1 or would also offer the possibility to study the consequences of a treatment with menadione or beta-lapachone directly *in vivo*. Additionally, this could include more detailed studies about the possible penetration of the blood-brain barrier by beta-lapachone or menadione after uptake into the organism and the effective concentrations reached in the brain.

As the bulk of NQO1 enzymatic activity is localized in the cytosol of rat primary astrocyte cultures (Ehrke *et al.* 2020) the metabolic WST1 assay developed by Stapelfeldt *et al.* (2017) is also mainly measuring the metabolic activity in the cytosol. However, using the same basic electron-shuttling system for the formation of WST1 formazan and an additional targeted expression of NQO1 into mitochondria, it might be feasible to also measure mitochondrial activity with this system. Even though there is a substantial basal NQO1 activity in the cytosol of astrocyte cultures already (Ehrke *et al.* 2020), additional NQO1 activity in mitochondria might be detectable, especially if cell were to be exposed to ketone-bodies like pyruvate to fuel mitochondrial metabolism. As an alternative, mitochondria could also be isolated by digitonin lysis (Minich *et al.* 2003) for metabolic studies involving the WST1 assay system and thereby removing cytosolic NQO1 activity completely that would otherwise provide a background signal. Also, for studies on intact cells either different cells than primary astrocytes could be used that contain lower basal activities of NQO1 or a variation of NQO1 could be targeted to the mitochondria that is less sensitive to dicoumarol due to the exchange of essential amino acids of the dicoumarol binding site (Ma *et al.* 1992, Chen *et al.* 1999). This would allow for the inhibition of cytosolic NQO1 activity by dicoumarol and would leave artificially introduced mitochondrial NQO1 activity intact.

To further study the importance of the chemical structure of menadione on the GSH oxidation, depletion in total GSx and the loss in vitality observed in astrocytes, astrocyte cultures could be exposed to a range of quinones that are similar to menadione in structure, like it has before been described for lawsone in chapter 3.2. This could help to elucidate the chemical reactivity of menadione that seems to be responsible for the GSH oxidation, GSx depletion and toxicity observed in astrocyte cultures after menadione treatment. Therefore, quinone-compounds (table 3.2) similar to menadione should be tested for their potential to induce GSH oxidation and toxicity in astrocyte cultures and should additionally be screened for their ability to substitute menadione in the WST1 assay.

Table 3.2: Quinones that could be investigated for their potential to induce GSH oxidation and toxicity in rat astrocyte primary cultures.

Structure	Name	Reference
	Menadione	(Klotz *et al.* 2014)
	Lawsone	(Klotz *et al.* 2014)
	Plumbagin	(Klaus *et al.* 2010)
	Juglone	(Klaus *et al.* 2010)
	2-methoxy-1,4-naphtoquinone	(Klaus *et al.* 2010)
	thymoquinone	(Krylova *et al.* 2019)

3.5 References

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