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RESEARCH

Ferroptosis is Involved in Acetaminophen Induced Cell Death

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Abstract The recently described form of programmed cell death, ferroptosis can be induced by agents causing GSH depletion or the inhibition of GPX4. Ferroptosis clearly shows distinct morphologic, biochemical and genetic features from apoptosis, necrosis and autophagy. Since NAPQI the highly reactive metabolite of the widely applied analgesic and antipyretic, acetaminophen induces a cell death which can be characterized by GSH depletion, GPX inhibition and caspase independency the involvement of ferroptosis in acetaminophen induced cell death has been investigated. The specific ferroptosis inhibitor ferrostatin-1 failed to elevate the viability of acetaminophen treated HepG2 cells. It should be noticed that these cells do not form NAPQI due to the lack of phase I enzyme expression therefore GSH depletion cannot be observed. However in the case of acetaminophen treated primary mouse hepatocytes the significant elevation of cell viability could be observed upon ferrostatin-1 treatment. Similar to ferrostatin-1 treatment, the addition of the RIP1 kinase inhibitor necrostatin-1 could also elevate the viability of acetaminophen treated primary hepatocytes. Ferrostatin-1 has no influence on the expression of CYP2E1 or on the cellular GSH level which suggest that the protective effect of ferrostatin-1 in APAP induced cell death is not based on the reduced metabolism of APAP to NAPQI or on altered NAPQI conjugation by cellular GSH. Our results suggest that beyond necroptosis and apoptosis a third programmed cell death, ferroptosis is also involved in acetaminophen induced cell death in primary hepatocytes.

Keywords Acetaminophen · Hepatotoxicity · Ferroptosis · Necroptosis · Glutathione

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Introduction

Ferroptosis is a recently described form of programmed cell death [1]. It is morphologically, biochemically and genetically distinct from apoptosis, necrosis and autophagy and it shows iron-dependency. It has been induced (and described) first time by the addition of the small molecule erastin to oncogenic RAS-mutant cell lines [1]. Erastin treatment caused a marked depletion in the glutathione (GSH) content of the cells. GSH depletion by erastin has been necessary for its lethality since ferroptotic cell death could be prevented by the supplementation of the culture medium with GSH or N-acetylcysteine (NAC) [2]. The further characteristic feature of ferroptotic cell death was the increased levels of lipid hydroperoxides [2]. The inhibition of different members of the antioxidant network such as the superoxide dismutase (SOD), thioredoxin reductase or catalase has not depleted the GSH or induced ferroptosis suggesting that unique biochemical changes downstream of GSH depletion should have been responsible for the selective induction of ferroptosis [2]. Finally it was revealed that the depletion of GSH the cofactor for glutathione peroxidase 4 (GPX4) caused the accumulation of peroxides. The generation of lipid ROS and the induction of ferroptosis could be observed by the direct inhibition or by the knockdown of GPX4 [2]. On the base of these observations ferroptosis inducers have been classified into two classes: class I agents that involve GSH depletion and class II agents that trigger ferroptosis through inhibition of GPX4 [3].

Beyond the erastin induced cell death of tumour cells glutamate-induced cell death in organotypic rat brain slices could also be inhibited with the ferroptosis inhibitor ferrostatin-1 [1]. It is not so surprising since the prolonged exposure to high concentrations of extracellular glutamate promotes oxidative toxicity in cells by inhibition of the cystine/glutamate antiporter [4] decreasing the cystine uptake, which lowers the intracellular level of GSH as it has been observed in tumour cells due to erastin treatment [1]. Furthermore recently direct in vivo evidence has been given that ferroptosis is not only limited to specific tumour cells treated with ferroptosis inducers, but GPX4 could prevent premature death of mice by actively restraining the ferroptotic machinery in kidney tubular cells [3].

On the base of the above observations the involvement of ferroptosis has been emerged in acetaminophen (APAP) induced cell death. Why has this assumption been seemed logical? The major part of APAP is conjugated with glucuronic acid or with sulphate and excreted however a small fraction is metabolized by cytochrome P450 enzymes (mainly by Cyp2E1) to N-acetyl-p-benzoquinone imine (NAPQI). This highly reactive metabolite reacts rapidly with GSH causing extensive GSH depletion [5–7]. After GSH is depleted, NAPQI reacts with cellular proteins and forms APAP adducts [8]. By the presently accepted hypothesis, NAPQI formation and protein binding, especially to mitochondrial proteins, is an important initiating event of cell death. In addition APAP administration results in a 60 % reduction in GPX activity [9]. Furthermore no caspase activation could be observed after APAP overdose [10] neither caspase inhibitors showed any protection [10–13].

Summarily APAP induces a cell death which can be characterized with GSH depletion, GPX inhibition and caspase independency. Thus the potential role of ferroptosis and ferroptosis inhibitors have been investigated in APAP induced cell death.

Materials and Methods

Materials

Ethylene glycol tetraacetic acid (EGTA), type IV collagenase, acetaminophen (APAP), ferrostatin-1, necrostatin-1, α-tocopherol, MTT were obtained from Sigma-Aldrich, St. Louis, MO. Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS) and antibiotic/antimycotic were purchased from Invitrogen™. All other chemicals were of analytical grade.

HepG2 Cell Culture

HepG2 cells were cultured in a cell culture incubator (Thermo Scientific Forma™ Series II 3111) at 37 °C, 5 % CO_2, 100 % relative humidity based on to ATCC guidelines. Briefly the cells were maintained in DMEM supplemented with 10 % FBS, 1 % antibiotic/antimycotic (complete growth medium) and were subcultured before reaching 100 % confluence usually in a 1:4 ratio.

Isolation and Culture of Primary Mouse Hepatocytes

Male NMRI mice (23–25 g) were purchased from Charles River (Gödöllő, Hungary). Hepatocytes were prepared by a three-step perfusion procedure. The liver was first flushed with Ca^{2+} free Earle's balanced salt solution (EBSS) containing EGTA, then with the same buffer without chelating agent and finally with EBSS containing Ca^{2+} and type IV collagenase. Perfusions were carried out at 37 °C, pH 7.4 as described by Jemnitz et al. [14]. Hepatocytes were then seeded at a density of 3×10^4 cells/well on a 96 well plate (Becton, Dickinson and Company) precoated with rat tail collagen type I solution. Cells were cultured in Williams Medium E containing 5 % fetal calf serum, 100 nM insulin, 2.5 µg/ml amphotericin B, 0.1 mg/ml gentamicin, 30 nM Na_2SeO_3, and 0.1 µM dexamethasone. Calf serum and amphotericin B were present for the first 24 h then omitted. Cells were maintained at 37 °C in a humidified atmosphere of 95 % air+5 % CO_2. 24 h after seeding, hepatocytes were treated as indicated.

Treatment of HepG2 Cells and Isolated Primary Mouse Hepatocytes

The cells were seeded homogenously in either 96 or 6 well plates. 96 well plates were used for the determination of effective drug concentrations, 8000 cells/well were seeded. The cells were seeded in complete growth medium and were incubated for 24 h, then replaced with the supplemented complete growth medium for the treatment. The growth medium was supplemented by the following agents: acetaminophen (APAP), ferrostatin-1 (Fer-1), α-tocopherol (α-TOC) and dehydroascorbic acid (DHA). DHA was prepared by halogenic oxidation of ascorbic acid following the method of Washko et al. [15].

Evaluation of Cell Viability

Cell viability was determined in 96 well plates. The growth medium from the plate was discarded and replaced with growth medium supplemented with 1/10 volume 5 mg/ml MTT dissolved in complete growth medium. The plate was incubated with the supplemented MTT for 4 h in cell culture incubator then it was replaced with dimethylsulfoxide (DMSO) to dissolve the formazan crystals and incubated for 10 min at 37 °C. The absorbance was determined by microplate spectrophotometer (Thermo Scientific Multiskan™ GO) at 570 nm.

Reverse Transcription and Real-Time PCR Analysis of CYP2E1 mRNA Levels

Total RNA was isolated from 6 well plates containing 1.3 million cells pro well using innuPREP RNA Mini Kit (analytikjena). Reverse transcription was accomplished using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturers guidelines and protocol.

cDNA amplification has been done by a PikoReal Real-Time PCR System (Thermo Scientific) and Sensifast™ SYBR® No-ROX Kit (Bioline). The CYP2E1 cDNA transcript was amplified with the forward primer 5′-GTTGC CTTGC TTGTC TGGAT CG-3′ and reverse primer 5′-CTTGT GGTTC AGTAG CACCT CC-3′. For normalization simultaneous amplification of GAPDH cDNA was accomplished with the forward primer 5′-TCCAC TCACG GCAAA TTCAA CG-3′ and reverse primer 5′-TAGAC TCCAC GACAT ACTCA GC-3′ [16].

GSH Measurement

For the determination of cellular GSH monochlorbimane (mClB) derivatization followed by HPLC separation and fluorescent detection was used [17]. 6 well plates containing 1.3 million cells pro well were washed with PBS and 1 ml of Tris-buffer (20 mM, pH 8.0) was added which was supplemented with mClB to reach 1 mM final concentration. After a 40 min incubation in the dark the derivatization was stopped with the addition of 100 % trichloroacetic acid (TCA). The solution was centrifuged at 15,000 g for 10 min and the supernatant was used for GSH determination.

For separation a Waters 2690 HPLC was used with a Teknokroma® C18 reverse phase, d=5 μm column. Gradient elution was used as 0.25 % sodium-acetate (pH 3.5) and methanol. The detector was a Waters 2475 fluorescent detector with excitation and emission set to 395 and 477 nm respectively. Quantitation was achieved by measuring GSH standards.

Results

The Effect of Ferrostatin-1 on Acetaminophen Induced Cell Death in HepG2 Cells

It is well known that HepG2 cells practically do not express phase I enzymes [18] and therefore cannot form the reactive metabolite NAPQI. Consequently no GSH depletion nor protein adducts formation is occurred in these cells [19]. However the monitoring of viability of HepG2 cells on APAP and on APAP + ferrostatin-1 treatment can be a useful tool for the investigation of the potential role of ferroptosis in APAP induced cell death. As it was expected the HepG2 cells showed quite high resistance to APAP treatment (Table 1). Significant decrease of the viability could only be observed at extremely high (25 mM) APAP concentration. Ferrostatin-1 (Fer-1) in the earlier successfully applied concentrations (0.1 and 0.5 μM) [1] did not show any effect on the viability of HepG2 cells both in the presence and in the absence of APAP (Table 1).

The Effect of Different Ferroptosis, Necroptosis Inhibitors and Antioxidants on Acetaminophen Induced Cell Death in Primary Mouse Hepatocytes

Since the mechanisms of APAP toxicity in humans are similar to mice [20] primary mouse hepatocytes are frequently used to study mechanisms of APAP toxicity. Thus the effect of the selective ferroptosis inhibitor ferrostatin-1 has been investigated on isolated primary mouse hepatocytes both in the presence and in the absence of different concentrations of APAP. To have further insight into the more detailed mechanism of APAP induced cell death the effect of a potent and selective inhibitor of RIP1 kinase, necrostatin-1 (Nec-1) [21, 22], the water soluble antioxidant vitamin C (DHA) and the lipophilic antioxidant α-tocopherol (α-toc) have also been investigated together and separately.

All the investigated compounds could elevate the viability of APAP treated primary mouse hepatocytes (Table 2). The effects of both the ferrostatin-1 and the necrostatin-1 were

Table 1 The effect of acetaminophen and ferrostatin-1 treatment on the viability of HepG2 cells

Fer-1 concentration [μM]	APAP concentration [mM]			
	0	5	10	25
0	100,0±9,6	90,6±11,1	84,7±4,7*	50,8±3,1*
0,1	104,7±14,7	106,3±9,8	81,2±8,2*	54,4±6,7*
0,5	101,3±3,5	94,5±16,0	81,9±13,3	54,2±5,3*
1	98,8±1,0	109,9±15,7	82,9±8,0*	51,9±3,2*
5	103,9±5,8	91,0±14,6	80,8±7,1*	51,5±5,7*
10	89,4±5,8	94,9±10,8	72,7±7,9*	47,6±1,4*

*Significantly different with respect to APAP non-treated (APAP 0 mM) (P<0.05)

Table 2 The effect of different ferroptosis, necroptosis inhibitors and antioxidants on the viability of acetaminophen treated primary mouse hepatocytes

Addition	APAP concentration [mM]		
	0	10	20
Control	100,0±2,8	80,4±7,7*	19,9±2,7*
Fer-1 (1 μM)	96,7±2,0	85,7±1,9*#	40,7±3,0*#
Fer-1 (0,5 μM)	98,2±2,4	89,2±3,7*#	40,1±8,1*#
α-toc (1 nM)	104,3±0,3	86,4±6,5*	33,4±4,0*#
DHA (1 mM)	106,4±3,1	93,2±3,4*#	52,2±1,6*#
α-toc (1 nM)+DHA (1 mM)	108,8±2,5	88,3±0,9*#	53,9±3,9*#
Nec-1 (25 μM)	96,8±5,9	93,5±9,3#	37,7±4,6*#

*Significantly different with respect to APAP non-treated (APAP 0 mM) ($P<0.05$)

Significantly different with respect to control ($P<0.05$)

more significant at the higher 20 millimolar APAP dose (Table 2). The cell protective effect of DHA and the combination of DHA and α-tocopherol exceeded the effect of the pure α-tocopherol (Table 2). At the extremely high 25 mM of APAP concentration only these two (DHA and DHA+α-tocopherol) combinations could give some protection (Table 2).

The Effect of Ferrostatin-1 on the Expression of CYP2E1 and on Cellular GSH Level in Primary Mouse Hepatocytes

To have further insight into the mechanism of the effect of ferrostatin-1 on APAP induced cell death, primary hepatocytes have been isolated and performed cell-based in vitro assays. As the next step CYP2E1 expression levels have been compared to determine whether the protective effect of ferrostatin-1 against hepatic injury resulted from altered APAP metabolism. As it was expected APAP treatment caused significant elevation of the expression of CYP2E1 (Table 3.). However no significant differences could be detected in CYP2E1 expression levels in ferrostatin-1 treated and control or in APAP and APAP plus ferrostatin treated hepatocytes during APAP-induced hepatocyte death (Table 3.) In addition the total GSH levels have also been determined to assess the potential influence of ferrostatin-1 on the detoxification status of the cells especially on the conjugation of the highly reactive NAPQI by GSH. As it was also expected on the base of our previous results [17, 23] APAP treatment significantly depleted the cellular GSH pool (Table 4.). However it seems that ferrostatin-1 treatment did not have any influence on the cellular GSH level (Table 4.).

The Determination of the Composition of the Cell Protective DHA+α-Tocopherol Cocktail

Because of the observed excellent cell protective feature of DHA+α-tocopherol cocktail we would have liked to find the best composition of this cell protection mixture. Thus primary mouse hepatocytes have been treated with APAP in the presence of different concentrations of DHA and α-tocopherol. Surprisingly the best protective effect could be measured at the earlier applied composition of the DHA+α-tocopherol cocktail (Table 5).

Discussion

The recently described form of programmed cell death, ferroptosis [1] could be induced by agents causing GSH depletion or the inhibition of GPX4 [2]. Further characteristic features of ferroptotic cell death are the increased levels of lipid hydroperoxides [2] and caspase independency [1]. Since NAPQI the highly reactive metabolite of the widely applied analgesic and antipyretic, APAP induces a cell death which can be characterized with GSH depletion, GPX inhibition [5–7] and caspase independency [10–13] the involvement of ferroptosis in APAP induced cell death has been emerged.

In the first set of experiments, the viability of HepG2 cells has been investigated upon the addition of APAP and the specific ferroptosis inhibitor ferrostatin-1 in different concentrations (Table 1). HepG2 cells showed quite high resistance to APAP treatment (Table 1), significant decrease of the viability could only be observed at extremely high (25 mM) APAP concentration. Furthermore ferrostatin-1 failed to elevate the viability of HepG2 cells in the earlier successfully applied concentrations (0.1 and 0.5 μM) [1] independently of the applied APAP concentrations (Table 1). It was in good concordance with our

Table 3 The effect of ferrostatin-1 on CYP2E1 mRNA levels in primer mouse hepatocytes

Addition	Relative CYP2E1 mRNA level
Control	1.0±1.9
APAP (20 mM)	462.2±1.9*
APAP (20 mM)+Fer-1 (1 μM)	453.0±1.6*
Fer-1 (1 μM)	1.5±2.8

*Significantly different with respect to control ($P<0.05$)

Table 4 The effect of ferrostatin-1 on GSH levels in primer mouse hepatocytes

Addition	Relative GSH level [%]
Control	100.0±4.1
APAP (20 mM)	2.6±1.3*
APAP (20 mM)+Fer-1 (1 μM)	1.9±1.5*
Fer-1 (1 μM)	75.9±8.5*

*Significantly different with respect to control ($P<0.05$)

Table 5 The effect of different concentrations of DHA and α-tocopherol on the viability of acetaminophen treated primary mouse hepatocytes

DHA concentration [μM]	α-TOC concentration [nM]			
	0	1	50	500
0	100,0±4,7	124,9±7,1*	111,8±4,2*	83,0±4,0*
10	83,3±5,2*	100,2±4,8	118,4±6,7*	100,5±7,1
50	87,1±4,3*	104,7±5,5	131,7±1,8*	100,0±6,4
100	91,6±3,8*	102,4±5,8	127,8±5,8*	103,7±6,0
500	100,1±5,3	130,0±8,6*	113,5±3,5*	92,3±5,5*
1000	124,0±3,5*	151,4±5,0*	123,2±7,2*	85,5±4,0*

*Significantly different with respect to APAP treated (APAP 10 mM) but DHA (DHA 0 mM) and α-tocopherol (α-tocopherol 0 nM) non-treated ($P<0.05$)

expectations and with the earlier observations, since it is well known that HepG2 cells practically do not express phase I enzymes [18] which is essential for the formation of the highly reactive metabolite NAPQI, therefore for the depletion of GSH [19]. All these observations exclude the possibility of the involvement of ferroptosis in APAP induced cell death in HepG2 cells. This statement can be further strengthened by the observation that the only clinically approved antidote for APAP overdose, N-acetylcysteine, which is also highly effective in animal models, did not protect HepG2 cells [24]. At this point we should notice that ferroptotic cell death could also be prevented by the supplementation of the culture medium with GSH or N-acetylcysteine [2]. Although the HepG2 cells are less sensitive to APAP treatment it has been observed that HepG2 cells were killed by APAP (Table 1) [24, 25] but the mechanism is definitively different from primary liver cells that form the reactive metabolite NAPQI and mainly involves apoptosis [12].

As against to the HepG2 cells the mechanisms of APAP toxicity in humans are quite similar to mice therefore primary mouse hepatocytes are frequently used to study mechanism of APAP toxicity [20]. Thus the effect of the selective ferroptosis inhibitor ferrostatin-1 has been investigated on isolated primary mouse hepatocytes in the presence and in the absence of different concentrations of APAP. To have further insight into the more detailed mechanism of APAP induced cell death the effect of a potent and selective inhibitor of RIP1 kinase, necrostatin-1 (Nec-1) [21, 22] and the water soluble antioxidant vitamin C (DHA) and the lipophilic antioxidant α-tocopherol have also been investigated together and separately.

Although the inhibitory effect of ferrostatin-1 on APAP induced cell death was significant at 10 millimolar APAP concentration, more remarkable protective effect could be observed at higher APAP (20 mM) concentration (Table 2). Hence the present experiments demonstrate for the first time that the GSH depletion and GPX inhibition due to APAP overdose [5–7, 9] may cause ferroptotic cell death, which can be avoided by the selective ferroptosis inhibitor ferrostatin-1. Furthermore this observation clearly suggests that ferroptosis can be involved in APAP induced cell death. Actually there is nothing surprising in this observation since ferroptosis could be induced by different agents which deplete the cellular GSH or inhibit the activity of GPX4 [1–3]. Similar to ferrostatin-1, the RIP1 kinase inhibitor necrostatin-1 could elevate the viability of APAP treated primer hepatocytes (Table 2). This observation reinforces the results of Takemoto et al. [26] and Zhang et al. [27] who found that necrostatin-1 protected both primary hepatocytes and mice from APAP-induced cytotoxicity. Necrostatin-1 treatment could reduce the APAP-induced ROS production but did not have any effect on the expression of CYP2E1 or on APAP-induced GSH exhaustion [26]. Here we should notice that ferrostatin-1 has also no influence on the expression of CYP2E1 in primary mice hepatocytes (Table 3). Furthermore ferrostatin-1 treatment both in the absence and in the presence of APAP did not have any effect on the cellular GSH level (Table 4). These observations suggest that the protective effect of ferrostatin-1 in APAP induced cell death is not based on the reduced metabolism of APAP to NAPQI or on altered NAPQI conjugation by cellular GSH. Interestingly the depletion of the GPX4 substrate GSH due to BSO or erastin treatments efficiently triggered ferroptosis which could be prevented by necrostatin-1 and ferostatin-1 in Pfa1 fibroblast cells [3].

All these results (Table 2) suggest that beyond necroptosis [26, 27] and apoptosis [24, 25] a third programmed cell death, ferroptosis is also involved in APAP induced cell death in primary hepatocytes. Furthermore ferrostatin-1 beyond necrostatin-1 can also be a potential antidote for APAP induced hepatotoxicity.

Ferroptosis does not seem to be a liver specific phenomenon since it has also been involved in iron dependent cell death in cancer cells treated with specific compounds that block cystine import and glutathione production, as well as cell death in rat organotypic hippocampal brain slices treated with toxic concentrations of glutamate [1], which also blocks cystine import [4]. Furthermore recently direct in vivo evidence has been given that ferroptosis is not only limited to specific tumour cells treated with ferroptosis inducers, but GPX4 could prevent premature death of mice by actively restraining the ferroptotic machinery in kidney tubular cells [3].

Since the erastin induced (ferroptotic) cell death was suppressed by the lipophilic antioxidant α-tocopherol [2] the possible protective effect of α-tocopherol, the water soluble antioxidant vitamin C (DHA) and the combination of DHA and α-tocopherol was investigated. Interestingly DHA and the combination of DHA and α-tocopherol exceeded the protective effect of the pure α-tocopherol (Table 2). The synergetic effect of α-tocopherol, ascorbate (and glutathione) is reasonable since the three key antioxidants play an interdependent role on the electron transfer stage of the cell due to their recycling [28, 29]. The marked beneficial effect of these antioxidants can be explained by this synergetic effect, furthermore by scavenging the lipid and non-lipid ROS. Since the formation of excess ROS can trigger at least two programmed cell death, necroptosis [26] and ferroptosis [1,2], thus we aimed at the finding of the optimal ratio of DHA and α-tocopherol in this antioxidant cocktail. The best protective effect could be reached by the combination of 1 nM α-tocopherol and 1 mM DHA (Table 5).

Summarily on the base of our result the involvement of ferroptosis in APAP induced cell death is likely. This assumption is supported by the protective effect of ferrostatin-1 on APAP treated primary hepatocytes. Since depletion of GSH and/or the inhibition of GPX4 are inducers of ferroptosis APAP treatment could not trigger this recently discovered programmed cell death in HepG2 cells. Necessarily it is not disputed that APAP-induced cell death is predominantly necrotic. However we should calculate with ferroptosis in regard of APAP induced cell death and the relationship of programmed necrosis (necroptosis) and ferroptosis shell be investigate in the future experiments.

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

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