Cell. Mol. Life Sci. 64 (2007) 192–205
1420-682X/07/020192-14
DOI 10.1007/s00018-006-6456-1
© Birkhäuser Verlag, Basel, 2007

Cellular and Molecular Life Sciences

Research Article

Histidine-induced injury to cultured liver cells, effects of histidine derivatives and of iron chelators

U. Rauen*, S. Klempt and H. de Groot

Institut für Physiologische Chemie, Universitätsklinikum, Hufelandstr. 55, 45122 Essen (Germany),
Fax: +49 201 723 5943, e-mail: ursula.rauen@uni-duisburg-essen.de

Received 23 October 2006; accepted 21 November 2006
Online First 18 December 2006

Abstract. The amino acid histidine is an excellent buffer and is therefore included in several organ preservation solutions used in transplantation medicine. However, when used at concentrations as in these solutions, histidine has a marked injurious potential. Therefore, we here assessed the mechanism of histidine-induced cell injury and searched for ways to use the buffering power of histidine but avoid histidine toxicity. When cultured hepatocytes were incubated in HTK solution or in modified Krebs-Henseleit buffer containing 198 mM L-histidine at 37 °C, most cells lost viability within 3 h (LDH release 86 ± 7% and 89 ± 5%, respectively). This injury was accompanied by marked lipid peroxidation, and was strongly inhibited by hypoxia, by the antioxidants trolox, butylated hydroxytoluene and *N*-acetylcysteine and by the membrane-permeable iron chelators 2,2′-dipyridyl, 1,10-phenanthroline, LK 614, LK 616 and deferoxamine. Thus, histidine-induced cell injury appears to be mediated by an iron-dependent formation of reactive oxygen species. D-Histidine, imidazol and L-histidine methyl ester also elicited marked injury, while the *N*-substituted derivatives *N*α-acetyl-L-histidine and *tert*-butyl-oxycarbonyl-histidine and histidine-containing dipeptides showed almost no toxicity. Histidine toxicity, its iron dependence and the superiority of *N*α-acetyl-L-histidine were also evident during/after cold (4 °C) incubations. Therefore, we suggest the addition of iron chelators to histidine-containing solutions, and/or replacing histidine with *N*α-acetyl-L-histidine in organ preservation solutions.

Keywords. Histidine, organ preservation, iron, *N*α-acetyl-L-histidine, histidine derivatives, cold, hypothermia.

Introduction

In current clinical practice, solid organ grafts procured for transplantation are either submitted to ‘simple cold storage’ or – less often – are continuously perfused during the period between the procurement of the graft from the organ donor and the recipient operation. Grafts submitted to ‘simple cold storage’ are flushed with organ preservation solutions during procurement and then stored under hypothermic conditions without further perfusion. During this ischemia, tissue acidosis rapidly develops and therefore, efficient buffering has been a major issue in the development of organ preservation solutions [1, 2]. Although it is necessary to prevent severe acidosis, mild to moderate acidosis has been shown to provide protection against hypoxic injury, which is a major component of ischemic injury [3, 4]. To allow the development of some acidosis but prevent the development of severe acidosis, high concentrations of buffers with a p*K* value in the slightly acidic pH range – as compared to physiological extracellular values – (*i.e.* with a p*K* value around pH 6.8) would be ideal.

The amino acid histidine is an excellent buffer at slightly acidic pH values, and is therefore used as ‘physiological’ buffer in several organ preservation solutions such as in histidine-tryptophan-ketoglutarate (HTK) solution

* Corresponding author.

or Celsior solution at high concentrations. However, in a previous study histidine turned out to contribute decisively to preservation solution toxicity (manuscript submitted for publication). Unfortunately, the buffer used in other preservation solutions, phosphate buffer, also showed cytotoxicity when applied at high concentrations. As a high buffering capacity is essential for organ preservation solutions, and as histidine is an otherwise almost ideal buffer, we set out to assess the mechanism of histidine-induced cell injury and provide options to use the buffering power of histidine but avoid histidine toxicity.

Materials and Methods

Animals

Male Wistar rats (250–300 g) were obtained from the Zentrales Tierlaboratorium (Universitätsklinikum Essen). Animals were kept under standard conditions with free access to food and water. All animals received humane care in compliance with the German Law on the Protection of Animals and the institutional guidelines as well as with the 'Ethical Principles and Guidelines for Scientific Experiments on Animals' (as issued by the Swiss Academy of Medical Sciences), and permission for the use of the animals for liver cell isolations was obtained from the local authorities (Bezirksregierung Düsseldorf, Germany).

Chemicals

RPMI 1640 medium was obtained from Gibco BRL Life Technologies (Karlsruhe, Germany), and Leibovitz L-15 medium, fetal calf serum, 1,10-phenanthroline, 2,2′-dipyridyl, diethylenetriaminepentaacetic acid (DTPA), *N*α-acetyl-L-histidine, L-histidine methyl ester, butylated hydroxytoluene (BHT), *N*-acetyl-L-cysteine and resazurin were obtained from Sigma-Aldrich (Taufkirchen, Germany). *N*-(*tert*-Butyl-oxycarbonyl)histidine, L-glycyl-L-histidine, L-histidyl-L-glycine, β-alanyl-L-histidine (carnosine) and 1-methyl-L-histidine were purchased from Bachem (Weil am Rhein, Germany), 1,1,3,3-tetramethoxy-propane and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) from Fluka (Neu-Ulm, Germany). D-Histidine was obtained from ICN Biomedicals (Eschwege, Germany) and deferoxamine mesylate (Desferal®) from Novartis Pharma (Nuremberg, Germany). Gas mixtures were from Messer Griesheim (Krefeld, Germany). HTK solution (for composition see Table 1) and the new iron chelators LK 614 and LK 616 were kindly provided by Köhler Chemie (Alsbach-Hähnlein, Germany). All other chemicals used, including L-histidine, were purchased from Merck (Darmstadt, Germany).

Table 1. Composition of HTK solution.

Na^+	15 mM
K^+	10 mM
Mg^{2+}	4 mM
Ca^{2+}	0.015 mM
Cl^-	50 mM
α-Ketoglutarate	1 mM
L-Histidine	198 mM
Mannitol	30 mM
Tryptophan	2 mM
pH	7.2
Osmolarity	310 mOsm

Composition of the clinically used organ preservation solution histidine-tryptophan-ketoglutarate (HTK) solution. pH at 20 °C, calculated osmolarity.

Cell culture

Hepatocytes isolated from male Wistar rats [5] and a rat liver endothelial cell line [6] were used for experiments. Hepatocytes were seeded onto collagen-coated 12.5-cm^2 culture flasks or onto collagen-coated six-well cell culture plates (both from BD Biosciences, Heidelberg, Germany) and cultured in L-15 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 8.3 mM glucose, 0.1% bovine serum albumin, 14.3 mM $NaHCO_3$, 50 µg/ml gentamicin and 1 µM dexamethasone. At 2 h after seeding, adherent cells were washed three times with Hanks' balanced salt solution (HBSS) and supplied with fresh medium. Experiments were started 20–22 h after the isolation of the cells.

The endothelial cells were derived from the liver of a male Wistar rat as described previously [6]. Briefly, cells were isolated by collagenase perfusion and hepatocytes were removed by differential centrifugation. Endothelial cells were obtained from the nonparenchymal cell suspension by selective adherence. The cultured cells were identified as sinusoidal endothelial cells by electron microscopical demonstration of sieve plates in the primary cultures and in early passages. The cells were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin/50 µg/ml streptomycin and 1 µM dexamethasone. Subcultures were obtained by trypsinization. For the experiments, the cells were split 1 : 3, seeded onto collagen-coated 12.5-cm^2 culture flasks and used as confluent monolayer cultures on day 7 after subcultivation.

Experimental procedures

Experiments at 37 °C. Hepatocytes or liver endothelial cells were incubated in HTK solution, in Krebs-Henseleit (KH) buffer (supplemented with HEPES; 115 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 5.9 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 1.2 mmol/l NaH_2PO_4, 1.2 mmol/l Na_2SO_4,

2.5 mmol/l $CaCl_2$, 20 mmol/l HEPES, pH 7.4) or in modified KH buffers (containing 50, 76, 100 or 198 mM L-histidine; NaCl was reduced to maintain isoosmolarity) at 37 °C. Alternatively, cells were incubated in modified KH buffers containing histidine derivatives (Fig. 1); in most cases, these derivatives were used in the concentration of 198 mM, in selected cases (see results) also in a concentration of 100 mM, 76 mM or 50 mM (NaCl was reduced in all these modified buffers to maintain or approach isoosmolarity). The pH of the histidine- or histidine derivative-containing KH buffer was adjusted, as KH buffer, to pH 7.4. Experiments were performed under normoxic conditions; incubations in KH buffer or the modified KH buffers were exposed to 21% O_2/5% CO_2/74% N_2, incubations in HTK solution to room air. To part of the incubations, iron chelators (100 µM 2,2′-dipyridyl, 100 µM 1,10-phenanthroline or 100 µM DTPA, 1 mM LK 614 or 1 mM LK 616) or antioxidants (20 µM BHT, 40 mM *N*-acetylcysteine or 1 or 5 mM trolox) were added; solvent controls were included wherever solvents (ethanol) were used. Other cultures were preincubated with the iron chelator deferoxamine (10 mM in cell culture medium, 30-min preincubation at 37 °C).

One series of experiments was performed under hypoxic conditions. For these experiments, hepatocytes were washed three times with HBSS (37 °C) and then covered with HTK solution that had been saturated with 100% N_2, and then gently flushed with 100% N_2 through cannulae piercing the rubber stoppers of the flasks as described in [5]. The flasks were flushed again with 100% N_2 each time a sample was taken.

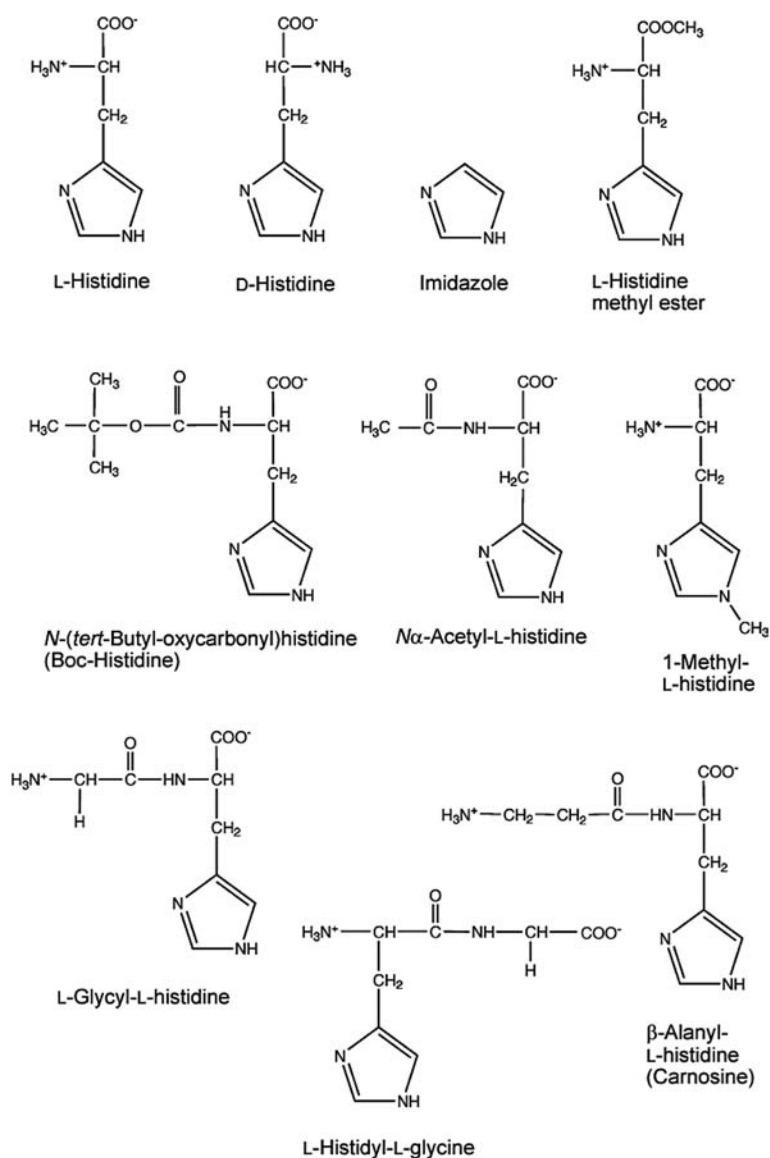

Figure 1. Histidine and histidine derivatives.

Experiments under hypothermic conditions. In an additional series of experiments, cells were exposed to hypothermia (4 °C). Part of the cultures was preincubated with the iron chelator deferoxamine (10 mM in cell culture medium, 30 min at 37 °C) prior to the start of the experiments. At the beginning of the experiments all cells were washed with HBSS (37 °C) and then covered with Krebs-Henseleit (KH) buffer, modified KH buffers (containing either 198 mM L-histidine or 198 mM Nα-acetyl-L-histidine), or HTK solution at room temperature. Cells were then incubated at 4 °C for 3, 5 or 12 h (hepatocytes) or for 24 h (liver endothelial cells). The incubations were performed in an atmosphere of 95% air/5% CO_2 (KH, modified KH; for cold incubations, these cell culture flasks/plates were placed in air-tight vessels that were flushed with the gas mixture) or under exposure to room air (HTK solution). After cold incubation, the cells were washed with cold HBSS, covered with cold cell culture medium and rewarmed to 37 °C in an incubator containing a humidified atmosphere of 95% air/5% CO_2.

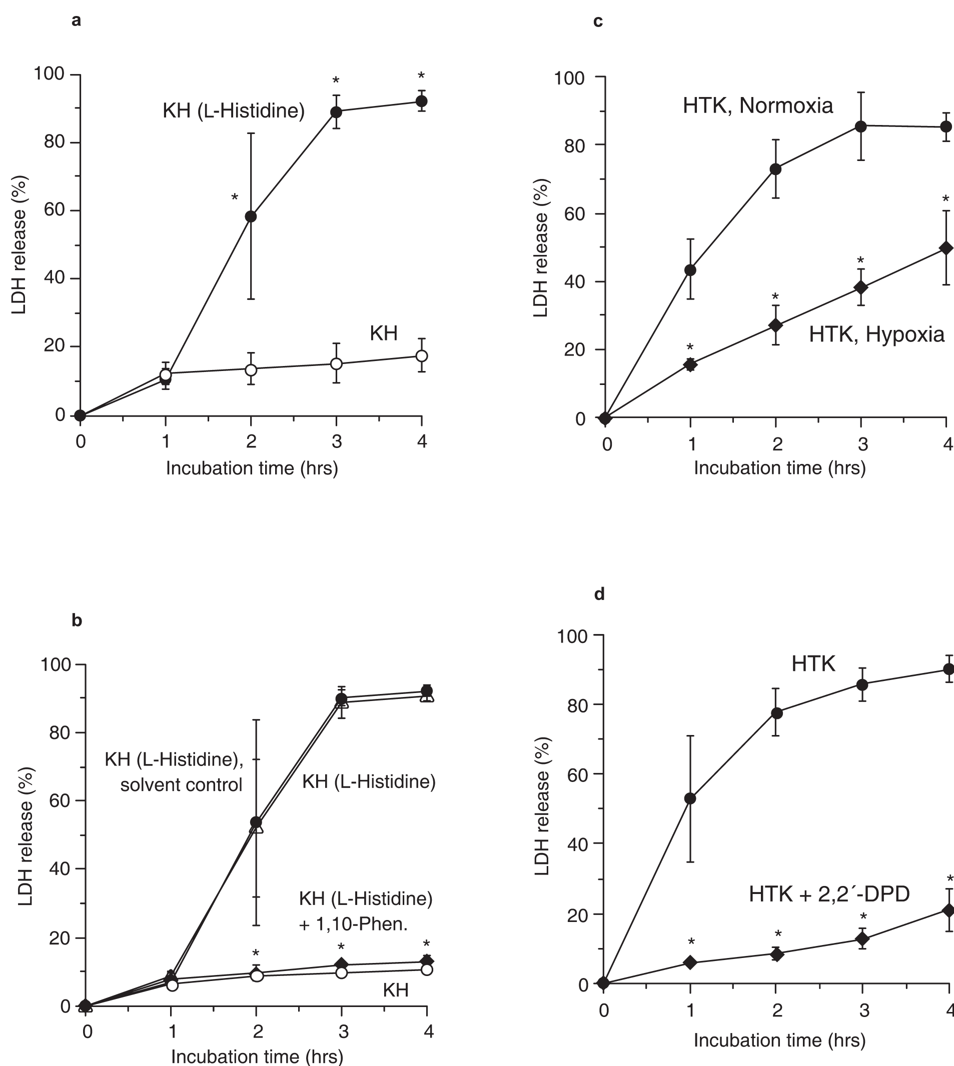

Figure 2. Histidine-induced cell injury and its inhibition by iron chelators or hypoxia. Monolayer cultures of rat hepatocytes were exposed to L-histidine in modified Krebs-Henseleit (KH) buffer (*a*, *b*), to histidine-tryptophan-ketoglutarate (HTK) solution (containing 198 mM L-histidine; *c*, *d*) or – for control – to KH buffer under otherwise physiological conditions (normoxia, 37 °C). Exposure in *a*, *b* was to 198 mM L-histidine [in modified KH buffer supplemented with 198 mM L-histidine; this buffer was rendered isoosmotic by the reduction of the NaCl concentration; KH (L-Histidine)]. To part of the cultures, an iron chelator was added, either 1,10-phenanthroline (1,10-Phen., 100 µM; *b*) or 2,2′-dipyridyl (2,2′-DPD, 100 µM; *d*). Where applicable, a solvent control (ethanol) was included (*b*). Other cultures were rendered hypoxic (*c*; all incubations in parts *a*, *b* and *d* were performed under normoxic conditions). The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH). Values shown are means ± SD of four experiments [some small SDs in (*b*) are masked by the symbols]. * Significantly different to KH buffer (*a*), to the respective incubation without inhibitor/solvent control (*b*, *d*) or to the respective normoxic incubation (*c*), $p < 0.05$.

Assays

Lactate dehydrogenase release. Extracellular, *i.e.* released, and cellular lactate dehydrogenase (LDH) activity were measured using a standard assay and released LDH activity was given as a percentage of total LDH activity [7].

Alamar Blue (resazurin reduction) assay. The Alamar Blue (resazurin reduction) assay was performed essentially as described in [8]. For this assay, cells were cultured in six-well culture plates. Control cells (time zero), cells incubated for 4 h at 37 °C in KH buffer, in modified KH buffers or in HTK solution with or without additives, or cells exposed to hypothermia (4 °C) in the different solutions and then rewarmed for 3 h were carefully washed with HBSS, and warm HBSS supplemented with 10 mM glucose was added. Cells were equilibrated for 15 min at 37 °C, then resazurin was added at a final concentration of 40 µM. The reduction of resazurin to the fluorescent resorufin was followed continuously at $\lambda_{exc.}$ = 560 nm, $\lambda_{em.}$ = 590 nm for 8 min (37 °C) using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). The rate of fluorescence increase (*i.e.* resazurin reduction) for cells exposed to experimental conditions is given as a percentage of the rate of fluorescence increase in control cells (in which the assay was performed at time zero).

Thiobarbituric acid-reactive substances. Thiobarbituric acid-reactive substances (TBARS) were determined in the supernatant incubation solution after warm or cold incubation as described previously [9]; 1,1,3,3-tetramethoxy-propane was used as a standard.

Determination of cellular ATP content. Cellular ATP content was determined enzymatically as described previously [6].

Statistics

All experiments were performed in duplicate and repeated three to five times. Data are expressed as means ± SD. Comparisons between experimental groups were performed using an analysis of variance with Dunnett or Bonferroni post hoc comparisons, as appropriate. A p value of <0.05 was considered significant.

Results

Mediation of histidine toxicity by reactive oxygen species

When cultured hepatocytes were incubated either in modified KH buffer containing 198 mM L-histidine or in HTK solution (containing 198 mM L-histidine) under otherwise physiological conditions, *i.e.* at 37 °C under normoxic conditions, more than 80% of hepatocytes lost viability (plasma membrane integrity) within 3 h (Fig. 2; 95% confidence intervals for LDH release at 4 h: 87–97% for cells incubated in L-histidine-containing KH buffer and 84–96% for cells incubated in HTK solution). The toxicity of HTK solution was strongly decreased under hypoxic conditions (Fig. 2c). As this might indicate an involvement of reactive oxygen species (ROS), the antioxidants *N*-acetylcysteine, BHT or trolox were added to HTK solution and strongly decreased HTK toxicity (Table 2). Both HTK-induced cell injury (data not shown) and histidine-induced cell injury (Table 3) were associated with marked lipid peroxidation.

Iron ions catalyze the conversion of ROS of low reactivity such as hydrogen peroxide to highly reactive species such as the hydroxyl radical, and are thus involved in various injuries mediated by ROS. Therefore, we tested whether iron chelators inhibit histidine-induced cell injury. The histidine-induced injury as studied in modified KH buffer as well as in HTK solution was completely/almost completely inhibited by the iron chelators 1,10-phenanthroline, 2,2′-dipyridyl and deferoxamine (Fig. 2b and d, Tables 2 and 3; upper limit of the 95% confidence interval for LDH release after 4-h incubation in L-histidine-containing KH buffer after pretreatment with deferoxamine: 44%). Similarly, the new iron chelators LK 614 (3,4-dimethoxy-*N*-methyl-benzhydroxamic acid) and LK 616 (2,3-dimethoxy-*N*-methyl-benzhydroxamic acid), two lipophilic hydroxamic acid derivatives, were strongly

Table 2. Inhibition of HTK toxicity by antioxidants and iron chelators.

	LDH release (%)
HTK	87 ± 5
HTK + 40 mM *N*-acetylcysteine	38 ± 12*
HTK + 1 mM Trolox	12 ± 4*
HTK + 5 mM Trolox	11 ± 3*
HTK + 100 µM butylated hydroxytoluene	14 ± 3*
HTK, solvent control (EtOH)	79 ± 5
HTK + 1,10-phenanthroline	13 ± 1*
HTK, solvent control (EtOH)	88 ± 8
HTK, preincubation with deferoxamine	30 ± 11*
HTK + 100 µM 2,2′-dipyridyl	13 ± 4*

Monolayer cultures of rat hepatocytes were exposed to HTK solution under otherwise physiological conditions (normoxia, 37 °C). To part of the cultures, antioxidants or iron chelators were added; other cultures were preincubated with the iron chelator deferoxamine (30-min preincubation with 10 mM deferoxamine in cell culture medium). Where applicable, a solvent control (ethanol, EtOH) was included; each solvent control applies to the condition immediately preceding it. The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH) after 4 h of incubation. Values shown are means ± SD of four experiments.
* Significantly different to the respective incubation without inhibitor/solvent control, $p < 0.05$.

Table 3. Histidine-induced injury and histidine-induced lipid peroxidation to cultured hepatocytes: effects of iron chelators.

	LDH release (%)	TBARS (nmol/10^6 cells)
KH	11 ± 1	0.7 ± 0.3
KH (198 mM L-histidine)	92 ± 2*	19.3 ± 7.2*
KH (198 mM L-histidine) + 100 µM 1,10-phenanthroline	13 ± 2**	1.2 ± 0.3**
KH (198 mM L-histidine), solvent control (EtOH)	91 ± 1	18.9 ± 4.1
KH (198 mM L-histidine), preincub. with deferoxamine	19 ± 16**	1.7 ± 1.1**
KH (198 mM L-histidine) + 100 µM 2,2′-dipyridyl	34 ± 13**	4.3 ± 3.2**
KH (198 mM L-histidine) + 100 µM DTPA	90 ± 4	16.8 ± 4.0
KH (100 mM L-histidine)	63 ± 26*	13.2 ± 6.3*
KH (100 mM L-histidine) + 100 µM 2,2′-dipyridyl	15 ± 8**	1.6 ± 0.8**
KH (100 mM L-histidine) + 1 mM LK614	15 ± 5**	0.8 ± 0.8**
KH (100 mM L-histidine) + 1 mM LK616	23 ± 18**	2.6 ± 2.4**
KH (100 mM $N\alpha$-acetyl-L-histidine)	15 ± 2#	1.5 ± 0.4#

Monolayer cultures of rat hepatocytes were exposed to L-histidine or $N\alpha$-acetyl-L-histidine in modified Krebs-Henseleit (KH) buffer or – for control – to KH buffer under otherwise physiological conditions (normoxia, 37 °C). Exposure was to either 100 mM or 198 mM L-histidine [in modified KH buffer supplemented with 100 or 198 mM L-histidine; this buffer was rendered isoosmotic by the reduction of the NaCl concentration, KH (L-histidine)], or to 100 mM of the histidine derivative $N\alpha$-acetyl-L-histidine (in modified KH buffer rendered isoosmotic as described above). To part of the cultures, a membrane-permeable iron chelator was added, either 1,10-phenanthroline, 2,2′-dipyridyl (2,2′-DPD, 100 µM) or one of the hydroxamic acid derivatives LK 614 or LK 616 (1 mM); where applicable, a solvent control (EtOH) was included; the solvent control applies to the condition immediately preceding it. Other cultures were preincubated with the iron chelator deferoxamine (30-min preincubation with 10 mM deferoxamine in cell culture medium). Additional cultures were exposed to the non-permeable iron chelator diethylenetriaminepentaacetic acid (DTPA, 100 µM). The occurrence of cell injury was assessed by the release of LDH after 4 h of incubation; as marker of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS) were determined after 4 h of incubation. Values shown are means ± SD of four experiments.
* Significantly different to KH buffer, $p < 0.05$.
** Significantly different to the respective incubation without inhibitor/solvent control, $p < 0.05$.
# Significantly different to modified KH buffer containing the same concentration L-histidine, $p < 0.05$.

protective (Table 3). While these membrane-permeable iron chelators provided complete protection, the non-permeable iron chelator DTPA did not affect histidine-induced cell injury at all (Table 3).

Similar protection by deferoxamine and 2,2′-dipyridyl against histidine-induced injury was observed when cellular metabolic/reducing activity was assessed using the Alamar Blue assay (Fig. 3a). Furthermore, when hepatocytes were exposed to L-histidine after pretreatment with deferoxamine or in the presence of 2,2′-dipyridyl, they were able to maintain ATP content, while ATP content strongly decreased during exposure to L-histidine in the absence of these iron chelators (Fig. 3b). Similar to their effect on cell viability, the iron chelators 2,2′-dipyridyl, 1,10-phenanthroline, deferoxamine and LK 614 completely and LK 616 strongly inhibited lipid peroxidation, but the non-permeable iron chelator DTPA did not offer any protection (Table 3).

ROS-mediated, iron-dependent histidine toxicity (in modified KH buffer as well as in HTK solution) was also observed in liver endothelial cells (Table 4). In these cells, the course of the injury was somewhat slower than in hepatocytes but the antioxidant *N*-acetylcysteine again provided partial protection and the iron chelator 2,2′-dipyridyl provided marked protection.

Use of histidine derivatives

Apart from its toxicity, histidine is, as outlined in the introduction, a substance of interest for organ preservation. Therefore, we tested whether histidine derivatives (Fig. 1) display a different toxicity. The histidine derivatives chosen for this purpose were the isomer D-histidine, the side-chain group imidazole, two derivatives modified at the amino group [$N\alpha$-acetyl-L-histidine, *N*-(*tert*-butyloxycarbonyl)histidine], a derivative modified at the carboxyl group (L-histidine methyl ester), a derivative modified at the side-chain group (1-methyl-L-histidine), and three histidine-containing dipeptides (dipeptides with the most simple amino acid glycine either at the amino or at the carboxyl end, and the dipeptide β-alanyl-L-histidine, which has been used in preservation studies before).

D-Histidine showed a slightly lower toxicity than L-histidine but was still fairly toxic (Fig. 4a). Imidazole was highly toxic (Fig. 4b). Similarly, L-histidine methyl ester exerted a very high toxicity (Table 5; data for a lower concentration of the derivative is shown to demonstrate the toxicity). Methylation in position 1 of the imidazole ring of L-histidine, in contrast, provided partial protection (Table 5; this derivative is very expensive and was therefore used at a lower concentration). Histidine-containing dipeptides also showed far lower toxicity than histidine itself: L-histidyl-L-glycine, L-glycyl-L-histidine and β-alanyl-L-histidine (carnosine) all showed low toxicity, L-glycyl-L-histidine no toxicity at all (Table 5). Also

derivatization at the amino group provided practically complete protection: *N*-(*tert*-butyl-oxycarbonyl)histidine (Boc-histidine) and $N\alpha$-acetyl-L-histidine exerted almost no toxicity (Fig. 4c and d; upper limit of the 95% confidence interval for LDH release after 4-h incubation in $N\alpha$-acetyl-L-histidine-containing KH buffer: 36%). This was evidently also true for a second concentration of $N\alpha$-acetyl-L-histidine (100 mM; Table 3). In addition, cells

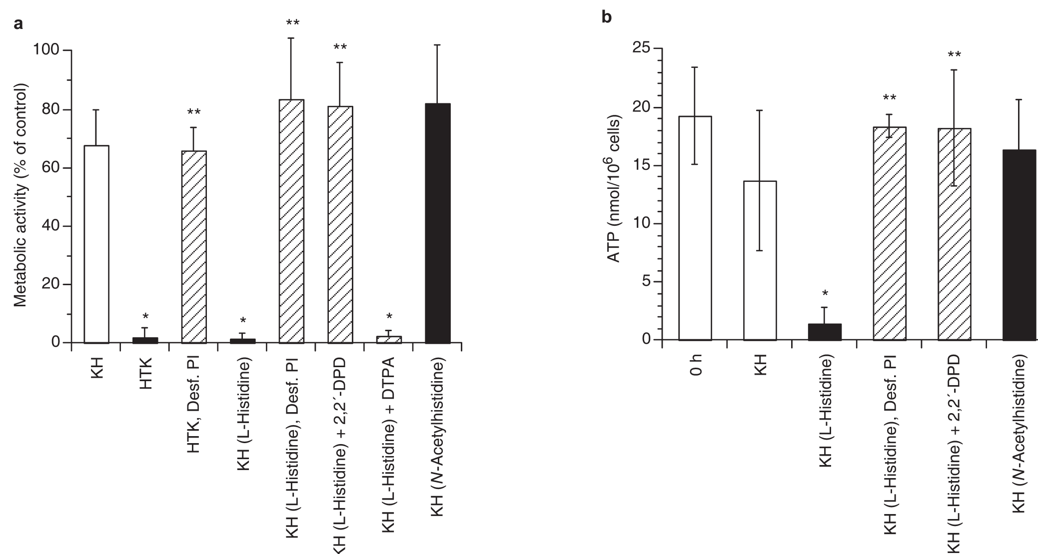

Figure 3. Histidine toxicity as assessed by functional parameters. Monolayer cultures of rat hepatocytes were exposed to L-histidine or to the histidine derivative $N\alpha$-acetyl-L-histidine in modified KH buffer, to HTK solution or – for control – to KH buffer under otherwise physiological conditions (normoxia, 37 °C). Exposure was to 198 mM L-histidine [in modified KH buffer supplemented with 198 mM L-histidine; this buffer was rendered isoosmotic by the reduction of the NaCl concentration; KH (L-Histidine)] or to 198 mM of the histidine derivative $N\alpha$-acetyl-L-histidine [in modified KH buffer without NaCl to approach isoosmolarity; KH (*N*-Acetylhistidine)]. KH buffer was devoid of substrates for energy metabolism. To part of the cultures, the membrane-permeable iron chelator 2,2′-dipyridyl (2,2′-DPD, 100 µM) or the membrane-impermeable iron chelator diethylenetriaminepentaacetic acid (DTPA, 100 µM) were added, other cultures were preincubated with the iron chelator deferoxamine (30-min preincubation with 10 mM deferoxamine; Desf. PI). Cellular metabolic/reducing activity was assessed by the Alamar Blue assay (*a*) or cellular ATP levels were determined enzymatically (*b*) after 4 h of incubation in the respective solutions. Baseline values (0 h) were determined at the beginning of the experiments. For the Alamar Blue assay, values are given as a percentage of the values for control cells assessed at time zero. Values shown are means ± SD of four experiments. * Significantly different to KH buffer, $p < 0.05$. ** Significantly different to the respective incubation without iron chelator, $p < 0.05$.

Table 4. Histidine- and HTK-induced injury to liver endothelial cells, its inhibition by antioxidants and iron chelators and effect of the histidine derivative $N\alpha$-acetyl-L-histidine.

	LDH release (%)	
	7 hrs	10 hrs
KH	6 ± 1	18 ± 3
KH (198 mM L-histidine)	56 ± 23*	82 ± 7*
KH (198 mM L-histidine) + 40 mM *N*-acetylcysteine	18 ± 8**	n.d.
KH (198 mM L-histidine) + 100 µM 2,2′-dipyridyl	12 ± 3**	n.d.
KH (198 mM $N\alpha$-acetyl-L-histidine)	n.d	38 ± 8*, #
HTK	83 ± 8*	n.d.
HTK + 40 mM *N*-acetylcysteine	46 ± 10**	n.d.
HTK + 100 µM 2,2′-dipyridyl	11 ± 1**	n.d.

Monolayer cultures of rat liver endothelial cells were exposed to L-histidine or $N\alpha$-acetyl-L-histidine in modified KH buffer, to HTK solution or – for control – to KH buffer under otherwise physiological conditions (normoxia, 37 °C). Exposure was to 198 mM L-histidine [in modified KH buffer supplemented with 198 mM L-histidine; this buffer was rendered isoosmotic by the reduction of the NaCl concentration, KH (198 mM L-histidine)], or to 198 mM of the histidine derivative $N\alpha$-acetyl-L-histidine [NaCl omitted to approach isoosmolarity; KH ($N\alpha$-acetyl-L-histidine)]. To part of the cultures, the antioxidant *N*-acetylcysteine or the iron chelator 2,2′-dipyridyl was added. The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH) after 7 and 10 h of incubation. Values shown are means ± SD of three to four experiments.

* Significantly different to KH buffer.

** Significantly different to the respective incubation without inhibitor, $p < 0.05$.

\# Significantly different to modified KH buffer containing the same concentration of L-histidine, $p < 0.05$.

n.d.: not determined.

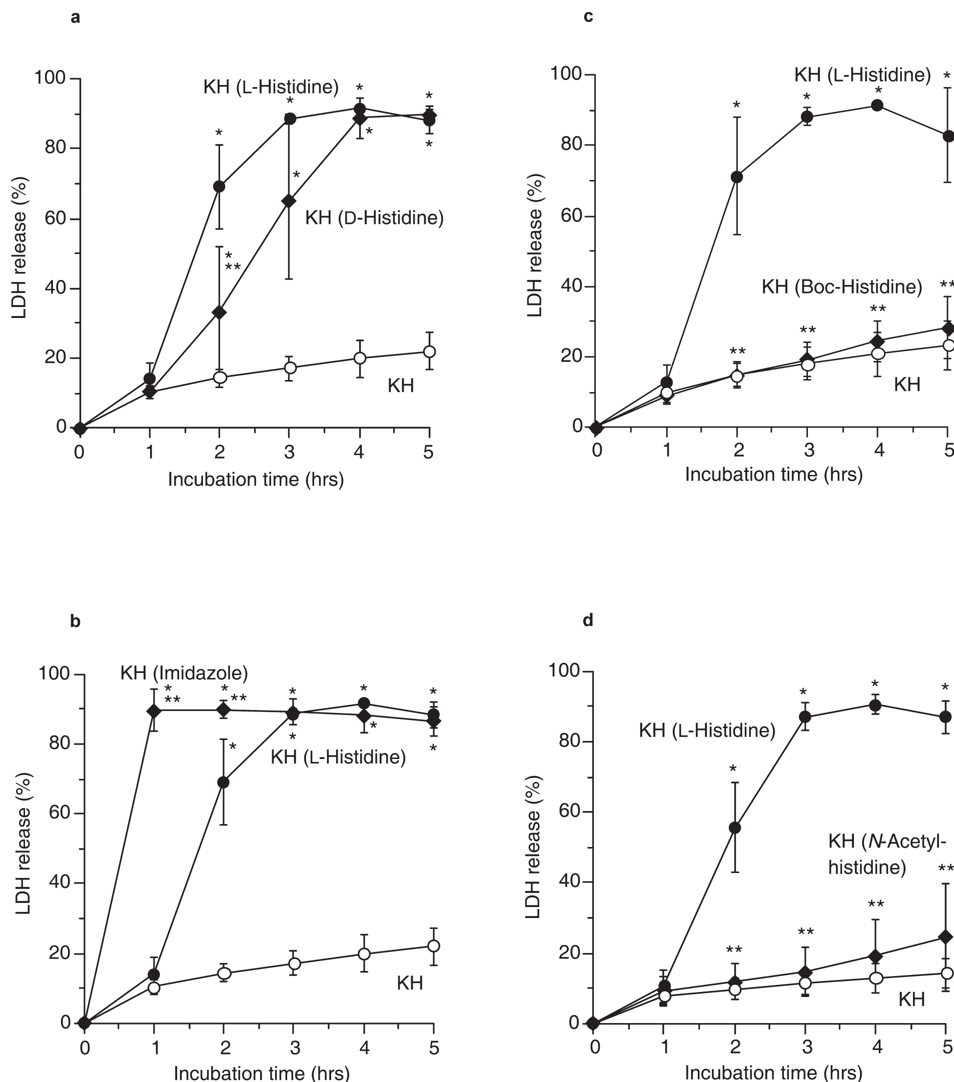

Figure 4. Effects of histidine derivatives. Monolayer cultures of rat hepatocytes were exposed to L-histidine or histidine derivatives in modified KH buffer or – for control – to KH buffer under otherwise physiological conditions (normoxia, 37 °C). Exposure was to 198 mM L-histidine [in modified KH buffer supplemented with 198 mM L-histidine; KH (L-Histidine)], to 198 mM D-histidine in modified KH buffer [KH (D-Histidine)] (*a*), to 198 mM imidazole [KH (Imidazole)] (*b*), to 198 mM *N*-(*tert*-butyl-oxycarbonyl)histidine [KH (Boc-Histidine)] (*c*), or to 198 mM $N\alpha$-acetyl-L-histidine [KH (*N*-Acetylhistidine)] (*d*). In all modified KH buffers the NaCl concentration was reduced in order to obtain or approach isoosmolarity. The occurrence of cell injury was assessed by the release of LDH. Values shown are means ± SD of four experiments. * Significant toxicity, *i.e.* significantly different to KH buffer, $p < 0.05$. ** Significantly different to modified KH buffer containing 198 mM L-histidine, $p < 0.05$.

exposed to the high concentration of 198 mM $N\alpha$-acetyl-L-histidine completely maintained their metabolic/reducing activity (Fig. 3a) and their ATP levels (Fig. 3b) over 4 h of incubation. In contrast to L-histidine, $N\alpha$-acetyl-L-histidine also did not give rise to lipid peroxidation (Table 3). Furthermore, $N\alpha$-acetyl-L-histidine proved to be far less damaging than L-histidine in liver endothelial cells (Table 4). The p*K* value of $N\alpha$-acetyl-L-histidine is, compared to histidine, shifted towards somewhat more alkaline values: while, at relevant ionic strength, the p*K* of histidine is 6.2 at 20 °C, the p*K* of $N\alpha$-acetyl-L-histidine is 7.2.

Histidine-induced injury during hypothermic incubation

When hepatocytes were exposed to hypothermia (4 °C) in KH buffer for 12 h, 30 ± 6% of cells lost viability during cold incubation and a further 54% during subsequent rewarming (Fig. 5a). In HTK solution and in modified KH buffer containing 198 mM L-histidine about 70% of cells already lost viability during cold incubation (Fig. 5a), while in modified KH buffer containing 198 mM $N\alpha$-acetyl-L-histidine only 28 ± 19% of hepatocytes lost viability during cold incubation and only a further 20% during rewarming (Fig. 5b). In all

Table 5. Histidine-induced injury to cultured hepatocytes: effects of histidine derivatives.

	LDH release (%)
KH	20 ± 5
KH (198 mM L-histidine)	92 ± 1*
KH (198 mM D-histidine)	89 ± 6*
KH (198 mM imidazole)	88 ± 5*
KH (198 mM *N*α-acetyl-L-histidine)	19 ± 11**
KH (198 mM Boc-histidine)	24 ± 6**
KH (198 mM L-histidyl-L-glycine)	36 ± 6*, **
KH (198 mM L-glycyl-L-histidine)	21 ± 10**
KH (198 mM *β*-alanyl-L-histidine)	39 ± 3*, **
KH (76 mM L-histidine)	91 ± 1*
KH (76 mM 1-methyl-L-histidine)	52 ± 32*, **
KH (50 mM L-histidine)	74 ± 18*
KH (50 mM L-histidine methyl ester)	90 ± 1*, **

Monolayer cultures of rat hepatocytes were exposed to 198, 76 or 50 mM L-histidine or histidine derivatives in modified KH buffer or – for control – to KH buffer under normoxic conditions at 37 °C. Modified KH buffers were rendered isoosmotic by the reduction of the NaCl concentration. The occurrence of cell injury was assessed by the release of LDH after 4 h of incubation. Values shown are means ± SD of three to four experiments.
* Significant toxicity, *i.e.* significantly different to KH buffer, $p < 0.05$.
** Significantly different to modified KH buffer containing the same concentration L-histidine, $p < 0.05$.

solutions, preincubation of the cells with the iron chelator deferoxamine provided protection: in KH buffer, protection was complete during cold incubation but only partial during rewarming, in all other solutions (HTK solution and modified KH buffers) protection by deferoxamine was complete during both, cold incubation and subsequent rewarming (Fig. 5a and b). Similar enhancement of cold-induced injury by histidine (HTK solution and modified KH buffer containing L-histidine) was observed when cell injury was assessed by monitoring cellular metabolic/reducing capacity (Fig. 5c); again, preincubation with deferoxamine provided protection against both cold-induced injury (as observed in KH buffer) and the enhancement of this injury by L-histidine. Furthermore, in this assay, *N*α-acetyl-L-histidine did not enhance cold-induced injury; cell injury in modified KH buffer containing *N*α-acetyl-L-histidine was even lower than in KH buffer.

Cold exposure of hepatocytes in KH buffer induced lipid peroxidation (Fig. 5d). Histidine exposure (HTK solution or modified KH buffer containing L-histidine) enhanced this lipid peroxidation, while *N*α-acetyl-L-histidine exposure did not enhance lipid peroxidation. Preincubation of hepatocytes with deferoxamine inhibited lipid peroxidation in all solutions.

Liver endothelial cells also suffered injury during cold incubation (Table 6). This injury was far stronger when cold incubation was performed in HTK solution or in modified KH buffer containing 198 mM L-histidine, while *N*α-acetyl-L-histidine only slightly enhanced cold-induced endothelial cell injury. Again, preincubating the cells with deferoxamine strongly inhibited cold-induced cell injury in all solutions.

Discussion

The results presented show clearly that histidine toxicity is mediated by an iron-dependent pathway and that the addition of a membrane-permeable iron chelator or appropriate derivatization of histidine can avoid this toxicity.

The iron involved in histidine toxicity appears to be intracellular, not extracellular iron, as membrane-permeable iron chelators (1,10-phenanthroline, 2,2′-dipyridyl, LK 614, LK 616 and the poorly, but still permeable deferoxamine), but not the impermeable iron chelator DTPA provided protection (Figs. 2 and 3, Tables 2–4). The intracellular iron involved in iron-dependent injurious processes belongs to the comparatively small pool of ‘chelatable iron’. The term ‘chelatable iron’ denotes cellular iron ions that are bound to low-molecular-weight ligands or loosely attached to macromolecules, and are thus accessible to exogenous chelators [10]. The iron ions of this pool (about 2–5 µM in cultured hepatocytes [10–12]) are, due to the abundance of cellular reducing equivalents, under physiological conditions predominantly present as ferrous iron (Fe^{2+}). However, these iron ions constantly redox-cycle between the ferrous and the ferric (Fe^{3+}) form: they are oxidized by hydrogen peroxide (H_2O_2) or molecular oxygen (O_2) and re-reduced by cellular reducing equivalents such as ascorbate or, more likely, NADH ([13, 14] and F. Petrat, H. de Groot, unpublished results). Most likely, histidine entering the cells chelates part of this iron. Histidine is a well-known iron chelator; however, its affinity for iron ions is moderate (stability constant for 2 : 1 complexes with Fe^{2+}: $\log \beta_2 = 10.43$ [15]), and is in the range where other cellular ligands also chelate iron. Thus, the formation of mixed complexes might also be possible. In contrast to strong iron chelators employed for cellular protection (which usually occupy all six coordination sites of iron ions and form redox-inactive complexes), many of the complexes with weaker ligands are redox-active and thus injurious (largely depending on the lacking occupancy of all coordination sites of iron ions and on the influence of the ligands on the redox potential of the iron ions). Apparently, the histidine entering the cells forms redox-active iron complexes with cellular chelatable iron. As a result, ROS are formed as indicated by lipid peroxidation. A similar mechanism has previously been suggested for the enhancement histidine exerts on hydrogen peroxide-induced DNA damage and on hydrogen peroxide-induced cell injury in some models [16, 17].

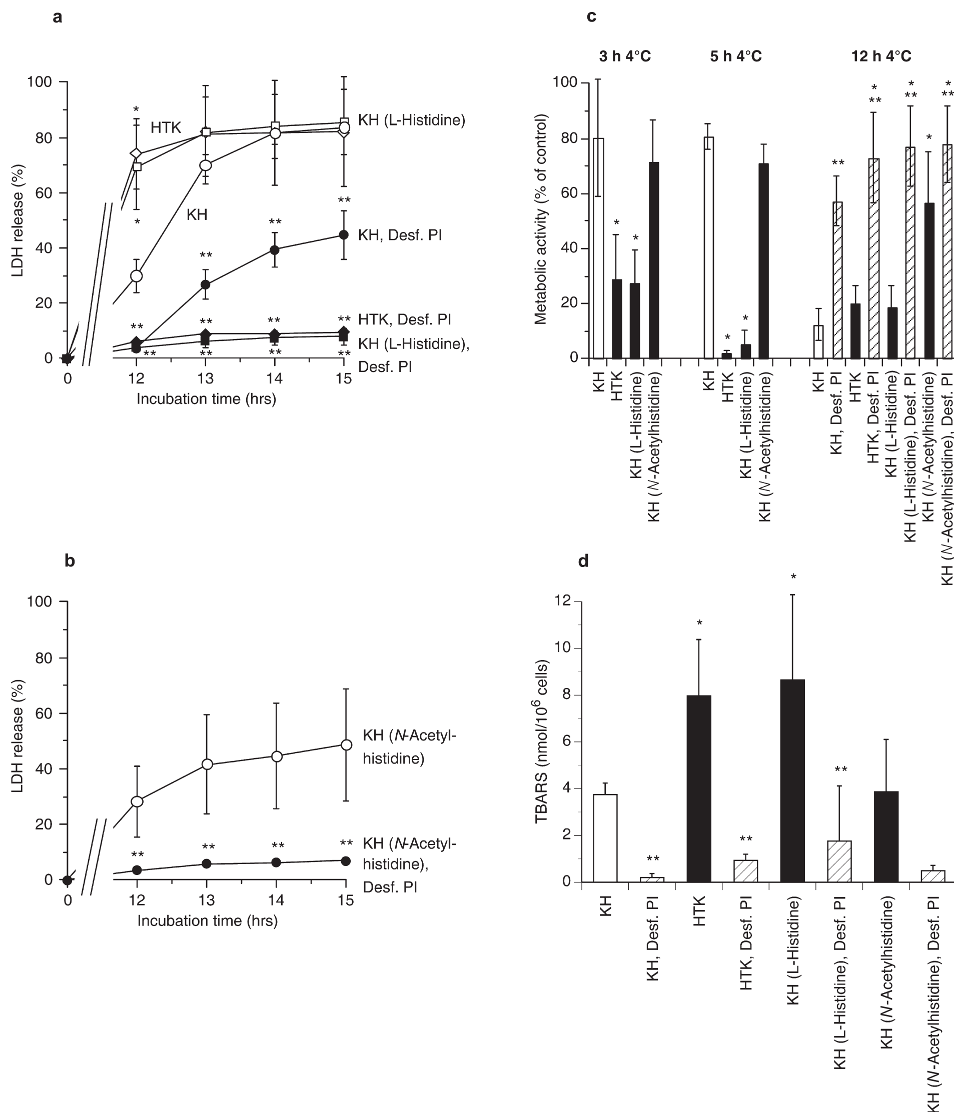

Figure 5. Histidine toxicity, its inhibition by the iron chelator deferoxamine and effect of $N\alpha$-acetyl-L-histidine during/after cold incubation of cultured hepatocytes. Monolayer cultures of rat hepatocytes were exposed to hypothermia (4 °C) in KH, in HTK solution or in L-histidine- or $N\alpha$-acetyl-L-histidine-containing modified KH buffers for 3–12 h and then rewarmed at 37 °C in cell culture medium for 3 h. L-Histidine-containing modified KH buffer contained 198 mM L-histidine [this buffer was rendered isoosmotic by the reduction of the NaCl concentration; KH (L-Histidine)], $N\alpha$-acetyl-L-histidine-containing modified KH buffer contained 198 mM of the histidine derivative $N\alpha$-acetyl-L-histidine [NaCl omitted to approach isoosmolarity; KH (*N*-Acetylhistidine)]. Part of the cultures were preincubated with the iron chelator deferoxamine (30-min preincubation with 10 mM deferoxamine in cell culture medium prior to cold incubation; Desf. PI). The occurrence of cell injury was assessed by the release of LDH after 12-h cold incubation and during subsequent rewarming (*a*, *b*). Cellular metabolic/reducing activity was assessed by the Alamar Blue assay after the 3 h rewarming period following different periods of cold incubation (*c*); for this assay, values are given as a percentage of the values for control cells not exposed to hypothermia and assessed at time zero. As marker of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS) were determined after the end of 12 h of cold incubation (*d*). Values shown are means ± SD of four to five experiments [some small SDs in (*a*) and (*b*) are masked by the symbols). * Significantly different from the incubation in regular KH buffer, $p < 0.05$. ** Significantly different from the respective incubation without iron chelator, $p < 0.05$.

Apart from its cytotoxicity, histidine is an excellent buffer, and therefore we tested diverse histidine derivatives to determine whether toxicity can be affected by derivatization. Imidazole, the side-chain group of histidine, alone was highly toxic with a very early occurrence of toxicity (Fig. 4, Table 5); this might be due to lysosomal accumulation of the weak amine followed by lysosomal disruption [18]. Histidine methyl ester also proved to be toxic; again, lysosomal accumulation of the compound might contribute, as the negative charge at the carboxyl group is lost and lysosomal hydrolysis of the ester might occur [18]. Methylation at position 1 of the imidazole

Table 6. Histidine toxicity, its inhibition by the iron chelator deferoxamine and effect of $N\alpha$-acetyl-L-histidine during/after cold incubation of cultured liver endothelial cells.

	LDH release (%)
KH	27 ± 11
KH, preincub. with deferoxamine	1 ± 1**
HTK	80 ± 9*
HTK, preincub. with deferoxamine	8 ± 5**
KH (198 mM L-histidine)	95 ± 1*
KH (198 mM L-histidine), preincub. with deferoxamine	32 ± 21**
KH (198 mM $N\alpha$-acetyl-L-histidine)	54 ± 14*
KH (198 mM $N\alpha$-acetyl-L-histidine), preincub. with deferoxamine	3 ± 2**

Monolayer cultures of rat liver endothelial cells were exposed to hypothermia (4 °C) in KH, in HTK solution or in L-histidine- or $N\alpha$-acetyl-L-histidine-containing modified KH buffers for 24 h and then rewarmed at 37 °C in cell culture medium for 3 h. L-Histidine-containing modified KH buffer contained 198 mM L-histidine [this buffer was rendered isoosmotic by the reduction of the NaCl concentration; KH (198 mM L-histidine)], $N\alpha$-acetyl-L-histidine-containing modified KH buffer contained 198 mM of the histidine derivative $N\alpha$-acetyl-L-histidine [NaCl omitted to approach isoosmolarity; KH (198 mM $N\alpha$-acetyl-L-histidine)]. Part of the cultures were preincubated with the iron chelator deferoxamine (30-min preincubation with 10 mM deferoxamine in cell culture medium prior to cold incubation). The occurrence of cell injury was assessed by the release of LDH during cold incubation and rewarming. Values shown are means ± SD of five experiments.
* Significantly different from the incubation in regular KH buffer, $p < 0.05$.
** Significantly different from the respective incubation without the iron chelator, $p < 0.05$.

ring provided some protection (probably by affecting cellular uptake and/or iron chelation) but toxicity was almost completely lost when derivatives substituted at the amino group and/or histidine-containing dipeptides were used. Of these derivatives, $N\alpha$-acetyl-L-histidine is particularly attractive as a buffer to be used in organ preservation solutions as it is a naturally occurring compound [19, 20], and as for an identically derivatized amino acid, *N*-acetylcysteine, vast experience in human beings exists. $N\alpha$-Acetyl-L-histidine's lack of toxicity (and probably also the lower toxicity of the dipeptides) is likely to be, at least partly, due to its decreased cellular uptake. In addition, an altered affinity for Fe^{2+} and/or altered redox activity of its iron complexes might play a role.

In the present study, histidine toxicity was first studied at 37 °C, while organ preservation solutions are used under hypothermic conditions. We decided to study histidine toxicity primarily at physiological temperatures to be able to differentiate the mechanism of histidine toxicity from other types of cell injury. However, evidence for histidine toxicity and for its iron-dependent mechanism could also be observed at 4 °C: cell injury during cold incubation in the histidine-buffered preservation solution HTK solution, as well as in histidine-supplemented KH buffer, was largely enhanced compared to KH buffer alone, and membrane-permeable iron chelators provided complete protection (Fig. 5, Table 6). However, hypothermia itself triggers an iron-dependent injury, as observed during/after cold incubation in KH buffer (Fig. 5, Table 6; compare incubations in KH buffer with and without deferoxamine pretreatment) and as described previously [7, 12, 21–26], and thus hampers proper study of the mechanisms of histidine toxicity. In cultured hepatocytes, hypothermia triggers an increase in cellular chelatable iron [12, 22, 23], and thus histidine and hypothermia are likely to act synergistically in the initiation of cell injury, which is evidenced by the strong enhancement of cold-induced cell injury in HTK solution or in histidine-containing KH buffer in hepatocytes as well as in liver endothelial cells. In contrast to L-histidine, $N\alpha$-acetyl-L-histidine, which in the warmth did not display toxicity, did not or hardly enhance cold-induced cell injury (Fig. 5, Table 6). Although it was very extensive, the combined injury elicited by histidine and by hypothermia was strongly/completely inhibited by the iron chelator deferoxamine.

In the experiments on hepatocyte injury during/after cold exposure (Fig. 5), the protection by the iron chelator was more marked in the L-histidine- or $N\alpha$-acetyl-L-histidine-containing solutions than in regular KH buffer (in which protection was only partial). This is due to the fact that the iron-independent component of cold-induced cell injury, which occurs in this cell type as a weaker, second-line injury and that becomes evident merely upon rewarming [22, 27], is mediated by chloride (manuscript submitted for publication), and was thus decreased in the solutions with low chloride concentrations (NaCl was decreased/omitted in L-histidine or $N\alpha$-acetyl-L-histidine-containing solutions for osmotic reasons). For the same reason, the injury occurring during rewarming after cold incubation was also largely reduced in the $N\alpha$-acetyl-L-histidine-containing modified KH buffer, compared to the regular KH buffer, in the absence of iron chelators (in the chloride-poor L-histidine-containing solutions, most cells were already dead before rewarming due to histidine toxicity).

Currently, continuous machine perfusion, one of the first methods to preserve kidney grafts, has gained renewed interest, particularly for kidney preservation, on the

background of organ shortage and the increased use of marginal organs [28–31]. Continuous machine perfusion allows the delivery of oxygen during storage and thus decreases ischemic injury. However, when continuous machine perfusion is performed at hypothermic temperatures, as it is usually done, the oxygen delivered is also likely to enhance free radical-mediated cold-induced cell injury. The use of histidine as a buffer should, under these conditions, be particularly detrimental. In fact, the conditions used in the cell culture experiments described in Fig. 5 and Table 6 simulate most closely the conditions of continuous hypothermic perfusion. The optimal preservation solutions for effective machine perfusion have not yet been established and modifications have recently been proposed [32–36]. Based on our data, we would suggest to include an iron chelator into these solutions and, in addition, to replace histidine by less toxic derivatives such as $N\alpha$-acetyl-L-histidine.

Besides its value as a buffer, histidine has been described to be an inhibitor of matrix metalloproteases [37]. However, the role of matrix metalloproteases in preservation injury is not clear yet: Upadhya and colleagues [38, 39] described a release of the matrix metalloproteases 2 and 9 by cultured liver endothelial cells exposed to hypothermia and a subsequent rounding and detachment of these cells, while we could not find evidence for a role of matrix metalloproteases in liver endothelial cell injury [40], and found injury and detachment of the endothelial cells to be a consequence of free radical-mediated processes [7].

As described in the introduction, other buffers used in organ preservation solutions, namely phosphate buffers, also have a cytotoxic potential, and, with regard to the pH range in which buffering is required, histidine has an almost optimal pK value (ideally, it should be slightly higher than the one of histidine, *i.e.* at about pH 6.8). Therefore, it would be ideal to be able to exploit the buffering capacity of histidine while at the same time avoiding histidine toxicity. The results presented in the current study offer two options to reach this goal: (i) use of histidine itself in combination with a membrane-permeable iron chelator, and (ii) use of $N\alpha$-acetyl-L-histidine (or histidine-containing dipeptides; Fig. 4, Table 5).

Both approaches offer advantages and disadvantages: in the first approach, histidine would still be able to exert toxicity if the iron chelator does not reach all cells/cellular compartments in sufficient concentrations to bind all chelatable iron ions safely. The second approach has, as far as dipeptides are concerned, the disadvantage that systemic side effects of the dipeptides (which may possess bioactivity [41]) would need to be excluded before any application can be considered. The use of $N\alpha$-acetyl-L-histidine appears favorable; however, its use in high concentrations requires a major redesign of preservation solutions as the charge of $N\alpha$-acetyl-L-histidine differs from the charge of histidine due to the loss of the positive charge of the amino group (histidine: zwitter ion or cation, $N\alpha$-acetyl-L-histidine: anion or zwitter ion, depending on the degree of protonation). For practical purposes, a combination of histidine (in decreased concentration) and $N\alpha$-acetyl-L-histidine plus the addition of a membrane-permeable iron chelator appears therefore the most feasible. With regard to the buffering capacity, $N\alpha$-acetyl-L-histidine is not inferior to histidine, and its pK value of 7.2 is fairly close to the desired value. As iron chelator, deferoxamine and/or the new lipophilic hydroxamic acid derivative LK 614 appear applicable.

In recent years, organ preservation solutions have – in addition to their use in classical organ transplantation – increasingly been used for the cold storage of cells and tissues [42–49]. It is likely, with the current efforts in the diverse fields of cellular therapies, as well as the advances in tissue engineering, that this use of preservation solutions will further increase in the future. As many of these applications require even longer storage and thus longer exposure periods, the avoidance of preservation solution toxicity as discussed above is of even greater importance for these fields, and the current study provides some options to reach this goal and improve the storage of diverse cells and tissues for biomedical and medical applications.

Acknowledgements. We thank Ms. N. Boschenkov, Ms. B. Lammers and Mrs. M. Brachvogel for their excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft (RA 960/1-2 and RA 960/1-3).

1 Belzer, F. O. and Southard, J. H. (1988) Principles of solid-organ preservation by cold storage. Transplantation 45, 673–676.
2 Blankensteijn, J. D. and Terpstra, O. T. (1991) Liver preservation: the past and the future. Hepatology 13, 1235–1250.
3 Gores, G. J., Nieminen, A. L., Fleishman, K. E., Dawson, T. L., Herman, B. and Lemasters, J. J. (1988) Extracellular acidosis delays onset of cell death in ATP-depleted hepatocytes. Am. J. Physiol. 255, C315-C322.
4 Bond, J. M., Herman, B. and Lemasters, J. J. (1991) Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. Biochem. Biophys. Res. Commun. 179, 798–803.
5 de Groot, H. and Brecht, M. (1991) Reoxygenation injury in rat hepatocytes: mediation by O_2^-/H_2O_2 liberated by sources other than xanthine oxidase. Biol. Chem. Hoppe-Seyler 372, 35–41.
6 Rauen, U., Hanssen, M., Lauchart, W., Becker, H. D. and de Groot, H. (1993) Energy-dependent injury to cultured sinusoidal endothelial cells of the rat liver in UW solution. Transplantation 55, 469–473.
7 Rauen, U., Polzar, B., Stephan, H., Mannherz, H. G. and de Groot, H. (1999) Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. FASEB J. 13, 155–168.
8 O'Brien, J., Wilson, I., Orton, T. and Pognan, F. (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 267, 5421–5426.
9 Noll, T., de Groot, H. and Sies, H. (1987) Distinct temporal relation among oxygen uptake, malondialdehyde formation, and low-level chemiluminescence during microsomal lipid peroxidation. Arch. Biochem. Biophys. 252, 284–291.

10 Petrat, F., de Groot, H., Sustmann, R. and Rauen, U. (2002) The chelatable iron pool in living cells: A methodically defined quantity. Biol. Chem. 383, 489–502.
11 Petrat, F., de Groot, H. and Rauen, U. (2000) Determination of the chelatable iron pool of single intact cells by laser scanning microscopy. Arch. Biochem. Biophys. 376, 74–81.
12 Rauen, U., Petrat, F., Li, T. and de Groot, H. (2000) Hypothermia injury/cold-induced apoptosis – evidence of an increase in chelatable iron causing oxidative injury in spite of low O_2^-/ H_2O_2 formation. FASEB J. 14, 1953–1964.
13 Lehnen-Beyel, I., de Groot, H. and Rauen, U. (2002) Enhancement of iron toxicity in L929 cells by D-glucose: accelerated (re-)reduction. Biochem. J. 368, 517–526.
14 Petrat, F., Paluch, S., Dogruöz, E., Dörfler, P., Kirsch, M., Korth, H. G., Sustmann, R. and de Groot, H. (2003) Reduction of Fe(III) ions complexed to physiological ligands by lipoyl dehydrogenase and other flavoenzymes *in vitro*: implications for an enzymatic reduction of Fe(III) ions of the labile iron pool. J. Biol. Chem. 278, 46403–46413.
15 Williams, D. R. (1970) Thermodynamic considerations in coordination. Part VII. Solubility of the histidine-H^+ system and stability constants, free energies, enthalpies, and entropies of the protonation of histidine and tryptophan and of formation of their manganese(II), iron(II), cobalt(II), nickel(II), copper(II) and zinc(II) complexes. J. Chem. Soc. (A), 1550–1555.
16 Cantoni, O. and Giacomoni, P. (1997) The role of DNA damage in the cytotoxic response to hydrogen peroxide/histidine. Gen. Pharmacol. 29, 513–516.
17 Tachon, P., Deflandre, A. and Giacomoni, P. U. (1994) Modulation by L-histidine of H_2O_2-mediated damage of cellular and isolated DNA. Carcinogenesis 15, 1621–1626.
18 Miller, D. K., Griffiths, E., Lenard, J. and Firestone, R. A. (1983) Cell killing by lysosomotropic detergents. J. Cell Biol. 97, 1841–1851.
19 Drescher, M. J. and Drescher, D. G. (1991) *N*-Acetylhistidine, glutamate, and β-alanine are concentrated in a receptor cell layer of the trout inner ear. J. Neurochem. 56, 658–664.
20 Baslow, M. H. (1997) A review of phylogenetic and metabolic relationships between the acylamino acids, *N*-acetyl-L-aspartic acid and *N*-acetyl-L-histidine, in the vertebrate nervous system. J. Neurochem. 68, 1335–1344.
21 Rauen, U. and de Groot, H. (1998) Cold-induced release of reactive oxygen species as a decisive mediator of hypothermia injury to cultured liver cells. Free Radic. Biol. Med. 24, 1316–1323.
22 Kerkweg, U., Li, T., de Groot, H. and Rauen, U. (2002) Cold-induced apoptosis of rat liver cells in University of Wisconsin solution: the central role of chelatable iron. Hepatology 35, 560–567.
23 Rauen, U. and de Groot, H. (2004) New insights into the cellular and molecular mechanisms of cold storage injury. J. Investig. Med. 52, 299–309.
24 Salahudeen, A. K. (2004) Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am. J. Physiol. Renal. Physiol. 287, F181-F187.
25 Huang, H. and Salahudeen, A. K. (2001) Iron release as a preventable mechanism for cold storage-induced rat kidney and human renal tubular cell injury. J. Am. Soc. Nephrol. 12, 859A.
26 Huang, H. and Salahudeen, A. K. (2002) Cold induces catalytic iron release of cytochrome P-450 origin: a critical step in cold storage-induced renal injury. Am. J. Transplant. 2, 631–639.
27 Rauen, U., Kerkweg, U., Weisheit, D., Petrat, F., Sustmann, R. and de Groot, H. (2003) Cold-induced apoptosis of hepatocytes: mitochondrial permeability transition triggered by nonmitochondrial chelatable iron. Free Radic. Biol. Med. 35, 1664–1678.
28 Rakhorst, G. and Ploeg, R. J. (2005) Revival of machine perfusion: new chances to increase the donor pool? Expert Rev. Med. Devices 2, 7–8.
29 van der Plaats, A., 'T Hart, N. A., Verkerke, G. J., Leuvenink, H. G. D., Ploeg, R. J. and Rakhorst, G. (2004) Hypothermic machine preservation in liver transplantation revisited: concepts and criteria in the new millennium. Ann. Biomed. Eng. 32, 623–631.
30 Brook, N. R., Waller, J. R. and Nicholson, M. L. (2003) Non-heart-beating kidney donation: current practice and future developments. Kidney Int. 63, 1516–1529.
31 Balupuri, S., Strong, A., Hoernich, N., Snowden, C., Mohamed, M., Manas, D., Kirby, J. and Talbot, D. (2001) Machine perfusion for kidneys: how to do it at minimal cost. Transpl. Int. 14, 103–107.
32 Bessems, M., Doorschodt, B. M., van Vliet, A. K. and van Gulik, T. M. (2005) Improved rat liver preservation by hypothermic continuous machine perfusion using polysol, a new, enriched preservation solution. Liver Transpl. 11, 539–546.
33 Bessems, M., Doorschodt, B. M., van Marle, J., Vreeling, H., Meijer, A. J. and van Gulik, T. M. (2005) Improved machine perfusion preservation of the non-heart-beating donor rat liver using Polysol: a new machine perfusion preservation solution. Liver Transpl. 11, 1379–1388.
34 Baicu, S. C., Taylor, M. J. and Brockbank, K. G. (2006) The role of preservation solution on acid base regulation during machine perfusion of kidneys. Clin. Transplant. 20, 113–121.
35 Lindell, S. L., Compagnon, P., Mangino, M. J. and Southard, J. H. (2005) UW solution for hypothermic machine perfusion of warm ischemic kidneys. Transplantation 79, 1358–1361.
36 Minor, T., Sitzia, M. and Dombrowski, F. (2005) Kidney transplantation from non-heart-beating donors after oxygenated low-flow machine perfusion preservation with histidine-tryptophan-ketoglutarate solution. Transpl. Int. 17, 707–712.
37 Upadhya, G. A. and Strasberg, S. M. (2000) Glutathione, lactobionate, and histidine: cryptic inhibitors of matrix metalloproteinases contained in University of Wisconsin and histidine/tryptophan/ketoglutarate liver preservation solutions. Hepatology 31, 1115–1122.
38 Upadhya, A. G., Harvey, R. P. C., Howard, T. K., Lowell, J. A., Shenoy, S. and Strasberg, S. M. (1997) Evidence of a role for matrix metalloproteinases in cold preservation injury of the liver in humans and in the rat. Hepatology 26, 922–928.
39 Upadhya, G. A. and Strasberg, S. M. (1999) Evidence that actin disassembly is a requirement for matrix metalloproteinase secretion by sinusoidal endothelial cells during cold preservation in the rat. Hepatology 30, 169–176.
40 Doeppner, T. R., Grune, T., de Groot, H. and Rauen, U. (2003) Cold-induced apoptosis of rat liver endothelial cells: involvement of the proteasome. Transplantation 75, 1946–1953.
41 Tomonaga, S., Tachibana, T., Takagi, T., Saito, E. S., Zhang, R., Denbow, D. M. and Furuse, M. (2004) Effect of central administration of carnosine and its constituents on behaviors in chicks. Brain Res. Bull. 63, 75–82.
42 Kunieda, T., Maruyama, M., Okitsu, T., Shibata, N., Takesue, M., Totsugawa, T., Kosaka, Y., Arata, T., Kobayashi, K., Ikeda, H., Oshita, M., Nakaji, S., Ohmoto, K., Yamamoto, S., Kurabayashi, Y., Kodama, M., Tanaka, N. and Kobayashi, N. (2003) Cryopreservation of primarily isolated porcine hepatocytes with UW solution. Cell Transplant. 12, 607–616.
43 Jay, T. R., Paget, M. B., Heald, K. A. and Downing, R. (2004) Are organ preservation solutions useful for the storage of isolated human islets? Transplant. Proc. 36, 1130–1132.
44 Lakey, J. R., Rajotte, R. V., Fedorow, C. A. and Taylor, M. J. (2001) Islet cryopreservation using intracellular preservation solutions. Cell Transplant. 10, 583–589.
45 Lee, K. W., Park, J. B., Yoon, J. J., Lee, J. H., Kim, S. Y., Jung, H. J., Lee, S. K., Kim, S. J., Lee, H. H., Lee, D. S. and Joh, J. W. (2004) The viability and function of cryopreserved hepatocyte

spheroids with different cryopreservation solutions. Transplant. Proc. 36, 2462–2463.

46 Mischiati, C., Puviani, A. C., Brogli, M., Guarniero, S., Sereni, A., Breda, L., Ricci, D., Galavotti, D., Morsiani, E. and Gambari, R. (2003) Modulation of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) gene expression in isolated porcine hepatocytes perfused within a radial-flow bioreactor after low-temperature storing. Int. J. Artif. Organs 26, 139–148.

47 Nguyen, T. H., Khakhoulina, T., Simmons, T., Morel, P. and Trono, D. (2005) A simple and highly effective method for the stable transduction of uncultured porcine hepatocytes using lentiviral vector. Cell Transplant. 14, 489–496.

48 Mathew, A. J., Baust, J. M., Van Buskirk, R. G. and Baust, J. G. (2004) Cell preservation in reparative and regenerative medicine: evolution of individualized solution composition. Tissue Eng. 10, 1662–1671.

49 Baust, J. M., Van Buskirk, R. and Baust, J. G. (2002) Modulation of the cryopreservation cap: elevated survival with reduced dimethyl sulfoxide concentration. Cryobiology 45, 97–108.