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New insights into generalized hepatoprotective effects of oleanolic acid: Key roles of metallothionein and Nrf2 induction

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ABSTRACT

Oleanolic acid (OA) is a natural triperpenoid that protects against a variety of hepatotoxicants such as carbon tetrachloride, cadmium, acetaminophen, and bromobenzene. To gain insight into the molecular mechanisms of this generalized hepatoprotection, genomic analysis was performed on mouse and rat livers after OA treatment. Mice and rats were given OA at a hepatoprotective dose (50 μmol/kg, s.c., daily for 4 days) and hepatic RNA was isolated, purified, and subjected to gene expression analysis. OA treatment produced changes in 5% of the genes on custom-designed mouse liver array and rat toxicology-II array. Changes in key gene expressions were further analyzed by real-time RT-PCR. OA treatment dramatically increased expression of hepatic metallothionein (*Mt*), and increased the expression of the nuclear factor E2-related factor 2 (*Nrf2*), NAD(P)H:quinone oxidoreductase 1 (*Nqo1*), heme oxygenase-1 (*Hmox1*), and glutamate–cysteine ligases (*Gclc* and *Gclm*). OA treatment also increased the expression of genes related to cell proliferation and suppressed the expression of several cytochrome P450 genes possibly to switch cellular metabolic energy to an acute-phase response. Hepatic MT protein was increased 60- and 15-fold in mice and rats, respectively, together with a 30% increase in mouse liver zinc. These gene expression changes, particularly the dramatic induction of MT and the Nrf2 signaling, occur with hepatoprotection doses of OA, and likely are important in the generalized protective effects of OA against hepatotoxicants.

1. Introduction

Oleanolic acid (OA) is a natural triterpenoid that exists widely in food and some medicinal herbs [1]. OA provides remarkable protection against acute and chronic liver injury in experimental models and has been used as an oral remedy for human liver disorders [1–3]. Pretreatment of experimental animals with non-toxic doses of OA can protect against acute hepatotoxicity produced by carbon tetrachloride [4,5], cadmium [6], acetaminophen [7] (Reisman et al. [34], SOT), bromobenzene, phalloidin, and D-galactosamine plus endotoxin [8]. OA produces increases in some, but not all, cellular antioxidants and produces a suppression of several drug-metabolizing enzymes, all of these have been proposed as mechanisms in OA-mediated hepatoprotection [9]. However, the molecular mechanisms of OA-mediated generalized hepatoprotection remain incompletely understood.

Microarray analysis has been used to profile gene expression patterns associated with zinc when given at hepatoprotective doses ([35], SOT). The notable gene expressions

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associated with the hepatoprotective level of zinc pretreatment were dramatic induction of metallothionein (MT). MT is a small, cysteine-rich protein playing important roles against oxidative damage [10], and induction of MT affects the magnitude and progression of toxicological insults [11]. Thus, induction of MT is important in zinc-induced generalized hepatoprotection, which could also be important for OA-induced generalized hepatoprotection. It would be of interest to profile the gene expression patterns following a low, hepatoprotective dose of OA, in both mice and rats, the two most commonly used experimental animal species in pharmacological studies.

In the present study, the gene expression patterns were examined in livers of mice or rats receiving a hepatoprotective dose of OA. The dose (50 μmol/kg, s.c.) and duration of treatment (once daily for 4 days) are documented to achieve the hepatoprotection against various hepatotoxicants without itself being overtly toxic to the liver [4–9]. This allows gene expression patterns responsible for subsequent hepatoprotection to be better evaluated. In addition, hepatic metallothionein protein and zinc content were also determined. The results clearly demonstrate that OA-induced similar gene expression changes in both mouse and rat livers, and these gene expression changes, particularly the dramatic induction of MT- and Nrf2-related genes, likely play a key role in the generalized hepatoprotection of OA against toxic insults.

2. Materials and methods

2.1. Chemicals

OA was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in 2% Tween-80 in saline. The Mouse Custom Atlas Array (600 liver-selective genes) and Rat Toxicology (II) Atlas Array (456 genes) were obtained from BD Biosciences (Clontech, Palo Alto, CA). [α-^{32}P]dATP was obtained from PerkinElmer Life Sciences (Boston, MA). Monoclonal antibodies against MT were purchased from Dako Cytometry (San Jose, CA). All other chemicals were commercially available and of reagent grade.

2.2. Animal treatments and sample collection

Adult male CD1 mice (25–30 g) and Sprague-Dawley rats (225–250 g) were purchased from the Animal Center of the Third Military Medical University (Chongqing, China) and maintained at the Animal Facilities of Zunyi Medical College with standard rodent chow and water *ad libitum* for at least 1 week prior to any treatments. Animal care was provided in accordance with the WHO Guideline of the Care and Use of Animals, and the study protocol was approved by the Institutional Animal Care and Use Committee.

Mice received s.c. injections of OA (50 μmol/kg, once daily for 4 days) in a volume of 10 ml/kg; rats received s.c. injections of OA (50 μmol/(kg day) for 4 days) in a volume of 3 ml/kg; while controls received the same volume of vehicle (2% Tween-80 in saline). Twenty-four hours after the last dose, animals were killed by CO_2 asphyxiation and the livers were removed, weighed, snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Microarray analysis

Total RNA was isolated from liver samples with TRIzol agent (Invitrogen, Carlsbad, CA), followed by purification with RNeasy columns (Qiagen, Valencia, CA). Approximately 5 μg of total purified RNA was converted to [α-^{32}P]dATP-labeled cDNA probe using MMLV reverse transcriptase and the Atlas customer array specific cDNA synthesis primer mix, and then purified with a NucleoSpin column (Clontech). The membranes were prehybridized with Expresshyb from Clontech for 2 h at 68 °C, followed by hybridization with the cDNA probe overnight at 68 °C. The membranes were then washed four times in 2× SSC/1% SDS, 30 min each, and two times in 0.1× SSC/0.5% SDS for 30 min. The membranes were then sealed with plastic wrap and exposed to a Molecular Dynamics Phosphoimage Screen. The images were analyzed densitometrically using AtlasImage software (Version 2.01). The gene expression intensities were first corrected with the external background and then globally normalized.

2.4. Real-time RT-PCR analysis

The levels of expression of the selected genes were quantified using real-time RT-PCR analysis. Briefly, purified RNA was reverse transcribed with MuLV reverse transcriptase and oligo-dT primers. The forward and reverse primer sequences for selected genes were designed with the ABI Primer Express software (Foster City, CA). The Power SYBR Green Master Mix (Applied Biosystems, Cheshire, UK) was used for real-time PCR analysis. The C_t values of the interested genes were first normalized with β-actin of the same sample, and then the relative differences between control and treatment groups were calculated and expressed as relative increases, setting control as 100%.

2.5. MT protein determination

Rodent liver and kidney were homogenized in 10 mM Tris–HCl buffer (1:5, w/v), followed by centrifugation at 20,000 × *g* for 10 min. Metallothionein concentrations in the cytosol were then determined by the cadmium/hemoglobin assay [12].

2.6. Hepatic zinc determination

A portion of the frozen liver (~100 mg) was completely digested in ultrapure nitric acid. Hepatic zinc contents were then determined using graphic furnace atomic absorption spectrometry (PerkinElmer AAanalst600, Norwalk, CT). Results are expressed as μg zinc/g wet weight liver.

2.7. Statistics

For microarray analysis, results from triplicate determinations from pooled liver samples were analyzed while individual liver samples ($n = 4$ for rats and $n = 5$ for mice) were used for real-time RT-PCR analysis. For comparison of gene expression level

between two groups, Student's t-test was performed. A level of $p < 0.05$ was considered significant in all cases.

3. Results

3.1. *Hepatic gene expression associated with hepatoprotective OA treatment in mice*

Oleanolic acid treatment of mice at a low, hepatoprotective dose (50 μmol/kg) for 4 days had no impact on body weights, and produced no any overt toxic manifestations (not shown). Liver weight and gross appearance were normal. Hepatic total RNA was extracted, purified, and subjected to Mouse Liver-Selective Custom Atlas Array and real-time RT-PCR analysis. Approximately 5% (30/588) of genes showed significantly ($p < 0.05$) altered expressions. Real-time RT-PCR confirmed and extended the initial microarray screenings, and selected results are categorized and shown in Table 1. Key data from real-time RT-PCR analysis are also illustrated in Fig. 1.

OA treatment dramatically increased the expression of metallothionein-1 (*Mt1*, 23 folds) and metallothionein-2 (*Mt2*, 29 folds). Expression of heat shock protein 70 (*Hsp70*) and signal transduction/activator 3 (*Stat3*) was increased approximately 2-fold. Other genes involved in acute-phase response, such as *c-jun*, early growth response protein 1 (*Egr1*), CXC chemokine (*Cxcl1*), and ceruloplasmin (*Cp*) were unaltered (Table 1).

Expressions of cellular antioxidant components, like nuclear factor erythroid 2-related factor 2 (*Nrf2*) and its downstream targets, including NAD(P)H:quinine oxidoreductase 1 (*Nqo1*), heme oxygenase-1 (*Hmox1*), glutamate–cysteine ligase (*Gclc*) and glutamate–cysteine modifier subunit (*Gclm*) were increased between 3- and 4-fold (Table 1 and Fig. 1). The expression of genes encoding GSH S-transferases (*Gstp1* and *Gstm3*) was not affected, while the expression of glutathione peroxidase 1 (*Gpx1*) and copper,zinc superoxide dismutase (*Sod1*) were unchanged or slightly increased.

The expression of tumor necrosis factor-α (*Tnfa*), its receptor *Tnfrsf1b*, and prostaglandin synthase Cox2 (*Ptgs2*) was slightly increased between 1.5- and 2.5-fold, while the expression of interleukin-1β (*Il1b*), interleukin-6 (*Il6*), and interleukin-10 (*Il10*) was not affected.

The expression of cytochrome P450 enzyme genes *Cyp2e1*, *Cyp2j5* and *Cyp2f2* was slightly suppressed, while the expression of *Cyp3a11* and *Cyp4a14* was unchanged. The expression of other metabolic enzyme genes, such as UDP-glucuronosyltransferase 1A1 (*Ugt1a1*) and betaine homocysteine methyltransferase (*Bhmt*), was also unaffected.

The expression of genes encoding cell growth and proliferation was also slightly increased, including cyclin D1 (*Ccnd1*), proliferate cell nuclear antigen (*Pcna*) and *c-myc*, but the expression of insulin-like growth factor-related genes was unaltered (not shown).

3.2. *Hepatic gene expression associated with hepatoprotective OA treatment in rat*

OA treatment of rats at a hepatoprotective levels (50 μmol/kg, s.c. for 4 days) had no impact on body weight, and did not produce overt toxic manifestations (not shown). Approximately 5% (24/456) of gene showed significantly ($p < 0.05$) altered expression. Real-time RT-PCR confirmed and extended the initial microarray screenings, and selected results are categorized and shown in Table 2.

OA treatment dramatically increased the expression of *Mt1* (6-fold) and *Mt2* (20-fold). Expression of *Hsp70* and alpha1-acid glycoprotein (*Agp*) was also increased by 2-fold. Other genes involved with the acute-phase response, such as *Egr1*, and CXC chemokine (*Cxcl5*), were unaltered.

Similar to that observed in mouse livers, increased expression of *Nrf2* (2.7-fold) and related genes like *Nqo1* (3.7-fold), and *Gclc* (3.4-fold), occurred after OA treatment in rats (Table 2 and Fig. 1). Expressions of both *Hmox1* (2-fold) and *Gclm* (1.6-fold) was tended to increase, but were not statistically significant. The expression of genes encoding GSH S-transferase P (*Gstp*) glutathione peroxidase 1 (*Gpx1*), liver catalase, and copper,zinc superoxide dismutase (*Sod1*) was unchanged or slightly increased.

The expression of cytokines (*Il6*, *Nos2*, *Il1b*) and *Tnfa* and its receptor *Tnfrsf1b* was unaffected by OA. The expression of cytochrome P450 enzyme genes *Cyp2e1* and *Cyp1a2* was suppressed, while the expression of *Cyp3a11* was unchanged. The expression of other metabolic enzyme genes, such as

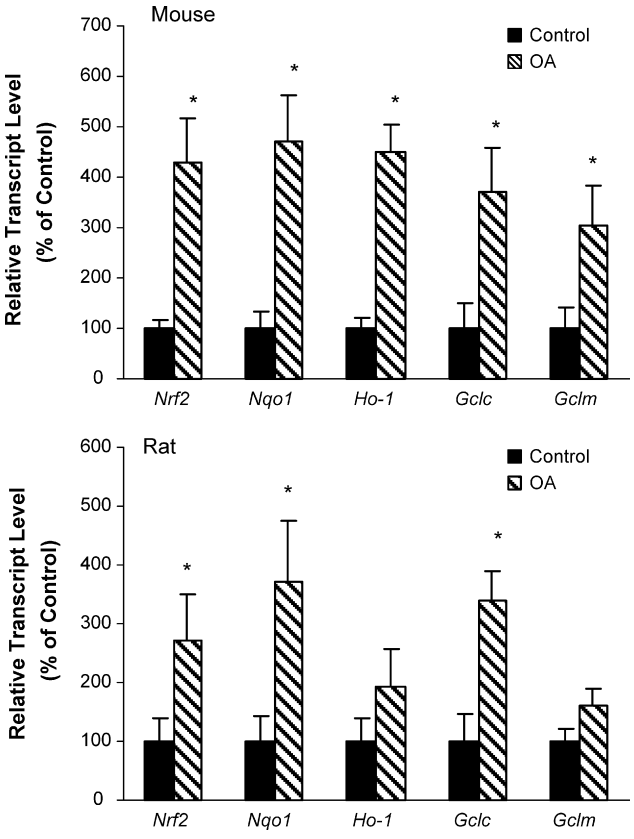

Fig. 1 – Effects of hepatoprotective level of OA on the expression of Nrf2 and downstream target genes. Mice and rats were treated with hepatoprotective dose (50 μmol/kg, s.c. daily for 4 days), and hepatic RNA was isolated and subjected to real-time RT-PCR analysis. Data are mean ± S.E.M. ($n = 4$ for rats and $n = 5$ for mice). Significantly different from controls $^*p < 0.05$.

Table 1 – Hepatoprotective levels of OA and hepatic gene expression changes in mice

Symbol	GeneBank#	Encoded proteins	Fold change (OA/control)	
			Array	RT-PCR
Acute-phase protein genes				
Mt1	BC027262	Metallthionein-1	n/a	**22.67**
Mt2	NM_008630	Meathiothinein-2	n/a	**29.40**
Hsp70	M35021	Heat shock protein 70	**3.01**	**2.00**
Stat3	U06922	Signal transduction/activator 3	**1.96**	**1.75**
c-jun	J04115	Jun oncogene	1.08	1.12
Egr1	M20157	Early growth response 1	1.63	1.43
Cxcl1	NM_008176	Chemokine (C-X-C motif) ligand 1	n/a	1.25
Cp	U49430	Ceruloplasmin	0.85	1.65
Antioxidant genes				
Nrf2	BC026943	Nuclear factor Nrf2	**7.71**	**4.30**
Nqo1	BC004579	NAD(P)H:quinone oxidoreductase 1	**1.75**	**4.70**
Hmox1	M33203	Heme oxygenase-1	**2.17**	**4.50**
Gclc	BC019374	Glutamate–cysteine ligase, catalytic	n/a	**3.70**
Gclm	NM_002061	Glutamate–cysteine ligase, modifier	n/a	**3.05**
Gstm3	J03953	Glutathione S-transferase mu3	1.02	1.65
Gstp1	D30687	Glutathione S-transferase pi	0.74	1.02
Gpx1	U13705	Glutathione peroxidase 1	**1.79**	**2.20**
Sod1	NM011434	Superoxide dismutase 1	1.07	**2.30**
Cytokines and inflammatory genes				
Tnf	XM_110221	Tumor necrosis factor-α	**2.35**	**2.40**
Tnfrsf1b	M59378	TNF receptor 1	**1.43**	**2.50**
Ptgs2	M64291	Prostaglandin synthase, Cox2	1.56	**2.80**
Il1b	NM_008361	Interleukin-1β	0.71	1.65
Il6	J03783	Interleukin-6	0.81	0.90
Il10	M37897	Interleukin-10	1.01	1.17
Metabolic enzyme genes				
Cyp2e1	L11650	Cytochrome P450 2E1	**0.69**	0.76
Cyp2j5	NM_010007	Cytochrome P450 2J5	**0.62**	**0.64**
Cyp2f2	M77497	Cytochrome P450 2F2	**0.73**	**0.50**
Cyp3a11	X60452	Cytochrome P450 3A11	0.78	1.36
Cyp4a14	Y11638	Cytochrome P4504A14	1.06	1.20
Cyp2f2	M77497	Cytochrome P450 2F2	0.73	0.50
Ugt1a2	D87866	UDP-glucuronosyltransferase 1A2	0.99	0.80
Bhmt	AF033381	Betaine homocysteine meT	1.13	0.80
Growth and cell proliferation genes				
Ccnd1	M64403	Cyclin D1	**3.11**	**2.55**
Pcna	X53068	Proliferate cell nuclear antigen	**2.12**	**3.10**
c-myc	X01023	c-myc proto-oncogene	**2.12**	1.89

CD1 mice were treated with OA (50 μmol/kg, s.c. daily for 4 days). Data are mean ± S.E.M. ($n = 3$ for microarrays and $n = 5$ for real-time RT-PCR analysis of individual mice). n/a means genes are not on the array. Bold indicates a significant difference from control ($p < 0.05$).

UDP-glucuronosyltransferase 1A1 (*Ugt1a1*) was not affected. The expression of genes encoding cell growth and proliferation was also slightly increased, including cyclin D1 (*Ccnd1*), *Pcna*, *c-myc* and *p21*.

3.3. OA treatment dramatically induces hepatic MT protein in mice and rats

Hepatic MT protein levels were determined by the cadmium/hemoglobin assay after treatments with hepatoprotective doses of OA. The OA treatment dramatically increased liver MT protein by 60-fold (1.5 μg/g vs. 90 μg/g) in mice, and by 15-fold (4.5 μg/g vs. 65 μg/g) in rats, respectively (Fig. 2, top panel). In addition, hepatic Zn concentrations were significantly increased by 30% (26.5 μg/g vs. 34.5 μg/g) in mice, but only slightly, but not significantly, increased in rats (35 μg/g vs. 39 μg/g) (Fig. 2, bottom panel).

4. Discussion

The present work demonstrated that OA treatment of mice or rats at hepatoprotective levels induced a consistent series of hepatic gene expression changes in both rats and mice. One of the most notable changes is the increase in MT expression, which occurs at both transcript and transcriptional levels. Significant increases of *Nrf2*- and Nrf2-related genes are also a consistent finding with hepatoprotective levels of OA in both mice and rats. In addition, a mild increase in expression of several adaptive response genes, including genes related to

Table 2 – Hepatoprotective levels of OA and hepatic gene expression changes in rats

Symbol	GeneBank#	Encoded proteins	Fold change (OA/control)	
			Array	RT-PCR
Acute-phase protein genes				
Mt1	J00750	Metallthionein-1	**6.50**	**5.99**
Mt2	M11794	Meathiothinein-2	n/a	**20.50**
Agp	J00696	Alpha1-acid glycoprotein	**2.68**	**1.50**
Hsp70	Z27118	Heat shock protein 70	**1.73**	1.51
Egr1	NM_012551	Early growth response 1	0.75	0.90
Cxcl5	U90448	Chemokine (C-X-C motif) ligand 5	n/a	**2.05**
Antioxidants and related genes				
Nrf2	AF037350	Nuclear receptor factor Nrf2	n/a	**2.75**
Nqo1	NM_017000	NAD(P)H dehydrogenase, quinone 1	**3.20**	**3.70**
Hmox1	J02722	Heme oxygenase-1	1.20	1.92
Gclc	NM_012815	Glutamate–cysteine ligase	**1.76**	**3.40**
Gclm	L22191	Glutamate–cysteine ligase, modifier subunit	1.56	1.60
Gstp	X02904	Glutathione S-transferase P	0.84	1.70
Gpx1	X12367	Glutathione peroxidase 1	0.81	1.80
Cat	M11670	Rat Liver catalase	1.11	**2.50**
Sod1	Y00404	Superoxide dismutase 1	1.25	1.50
Cytokines and inflammatory mediator genes				
Il6	J03783	Interleukin-6	1.03	1.05
Nos2	M87039	Inducible nitric oxide synthetase	1.22	2.01
Il1b	NM_031512	Interleukin-1β	0.90	1.65
Tnfa	X66539	Tumor necrosis factor-α	0.50	1.02
Tnfrsf1b	M63122	TNF receptor 1	1.42	1.41
Metabolic enzyme genes				
Cyp2e1	NM_031543	Cytochrome P450 2E1	**0.63**	**0.60**
Cyp1a2	M26127	Cytochrome P450 1A2	**0.40**	**0.60**
Cyp3a11	U09742	Cytochrome P450 3A11	1.10	1.00
Ugt1a1	NM_012683	UDP-glycosyltransferase 1A1	1.26	1.21
Cell proliferation-related genes				
Ccnd1	M64403	Cyclin D1	**1.82**	**2.00**
Pcna	Y00047	Proliferate cell nuclear antigen	**2.20**	**1.70**
c-myc	Y00396	c-myc oncogene	**1.85**	**2.30**
p21	L41275	p21, cip1, waf1	**1.90**	1.50

SD rats were treated with OA (50 μmol/kg, s.c. daily for 4 days). Data are mean ± S.E.M. ($n = 3$ for microarrays and $n = 4$ for real-time RT-PCR analysis of individual rat). n/a means genes are not on the array. Bold indicates a significant difference from control ($p < 0.05$).

acute-phase proteins, cellular antioxidants, and cellular proliferation, as well as a mild suppression of cytochrome P450-related genes, were also observed in both rodent species. Together these gene expression changes, particularly the dramatic increases in MT- and Nrf2-related signaling, occur in the absence of any OA-induced liver injury, and very likely are keys in the hepatoprotective effects of OA against a wide variety of hepatotoxicant insults.

MT is a low-molecular-weight, metal-binding protein that plays important roles in the detoxication of heavy metals, in the homeostasis of essential metals, and in the scavenging of free radicals [10]. Induction of MT by OA has been proposed to be a primary mechanism of protection against cadmium liver injury [6], and may play a role in protection against acetaminophen hepatotoxicity [7,13]. Indeed, induction of MT is an important adaptive mechanism affecting the magnitude and progression of toxic stimuli [11]. Thus, it can be concluded that the dramatic induction of MT is one of the mechanisms important for oleanolic acid-induced hepatoprotection.

However, MT induction alone is insufficient to fully explain OA-induced generalized hepatoprotection. For example, OA protects against carbon tetrachloride liver injury in MT-1/2 null mice, which is clearly independent of MT [14]. Thus, other mechanisms in addition to MT induction must also be involved. The Nrf2-signaling pathway has emerged as an important regulator of mammalian defense system for detoxication of toxic oxidants, which is mediated through the upregulation of *Nqo1* and other cellular antioxidants [15–19]. For instance, Nrf2 signaling functions in reducing the hepatotoxicity of carbon tetrachloride, acetaminophen, bromobenzene, and furosemide [19]. Thus, the induction of *Nrf2* and *Nqo1*, might well contribute to OA-induced generalized hepatoprotection.

The OA derivative CDDO-Im is a potent Nrf-2 activator [22–24], CDDO-Im-induced protection against LPS-induced toxicity and inflammation is mediated through Nrf2 activation [23]. It has been shown that OCCD-Im is a potent activator of Nrf2 signaling, which is mechanistically significant in protection against acetaminophen hepatotoxicity (Reisman et al. [34], SOT). In comparison, the synthetic OA derivative CDDO-Im is more potent and efficient than the parent compound in the induction of Nrf2 signaling.

Other OA-induced gene expression changes could also contribute to the adaptive responses or generalized hepatoprotection. For example, heat shock proteins could mediate

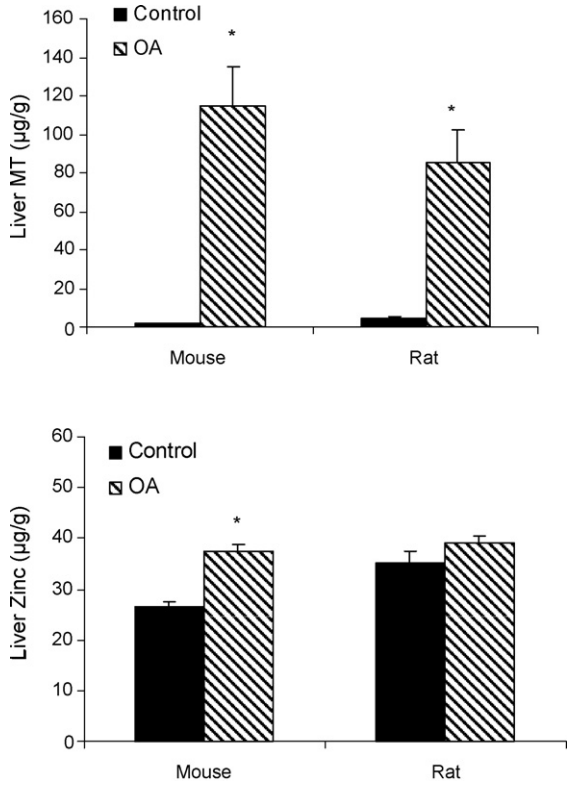

Fig. 2 – Hepatic MT and zinc contents. Mice and rats were treated with OA at a hepatoprotective level (50 μmol/kg, s.c. daily for 4 days), and hepatic MT protein and zinc were determined (see Section 2). Data are mean ± S.E.M. ($n = 4$ for rats and $n = 5$ for mice). Significantly different from controls ($^*p < 0.05$).

hepatic response to inflammatory stimuli [24], and HSP70-null mice are more susceptible to CCl_4 hepatotoxicity [25] and acetaminophen-induced liver injury [26]. Thus, OA-induced increases in the expression of *Hsp70* in mice and rat could be a part of a generalized protection mechanism. In agreement with the present observation, OA has been shown to suppress hepatic drug-metabolizing enzymes [9,27], and inhibition of CYP2E1 may well contribute to reduction of carbon tetrachloride hepatotoxicity [5]. All of these gene expression changes point towards the fact that multiple mechanisms are likely involved in the generalized hepatoprotection induced by OA.

Another interesting observation is the increased expression of genes related to liver proliferation or regeneration following OA treatment. The increases in liver proliferation-related genes, for example following zinc treatment, may contribute to adaptation responses, as the proliferating livers are resistant to hepatotoxicant insults [28]. Timely liver regeneration can prevent progression of injury by upregulation of toxicant-resistant proteins such as calpastatin [29]. It should also be noted that dramatic induction of MT by OA could also play an important role in liver regeneration, as MT is an important cellular modulator for liver regeneration soon after toxic insults [30].

It should also be kept in mind that OA can have both hepatoprotective and hepatotoxic effects [3,31]. Overdose of OA could be hepatotoxic or harmful, as ''dose makes a poison''. Another problem with OA administration is poor water solubility. Recently, the nanopreparation of OA has been used to improve its bioavailability, with increased hepatoprotective effects [32]. The low oral bioavailability of OA appears to be due to poor gastrointestinal absorption and extensive metabolic clearance [33]. In this regard, the synthetic OA derivative, CDDO-Im, has much better water solubility, bioavailability than the parent compound [20–22], and shows more pronounced biological effects [3] (Reisman et al. [34], SOT).

In summary, the current study demonstrated that hepatoprotective levels of OA in rat and mice evoked consistent pattern an array of gene expression changes, including dramatic increases in MT- and Nrf2-related gene expression, which provide new insight into the generalized hepatoprotective mechanisms of OA.

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