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Alteration of cardiac cytochrome P450-mediated arachidonic acid metabolism in response to lipopolysaccharide-induced acute systemic inflammation

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ABSTRACT

Cytochrome P450 (CYP) generated cardioprotective metabolites, epoxyeicosatrienoic acids (EETs), and cardiotoxic metabolites, hydroxyeicosatetraenoic acids (HETEs) levels are determined by many factors, including the induction or repression of the CYP enzymes, responsible for their formation. Therefore, we examined the effect of acute inflammation on the expression of CYP epoxygenases and CYP ω-hydroxylases in the heart, kidney, and liver and the cardiac CYP-mediated arachidonic acid metabolism. For this purpose, male Sprague–Dawley rats were injected intraperitoneally with LPS (1 mg/kg). After 6, 12, or 24 h, the tissues were harvested and the expression of CYP genes and protein levels were determined using real time-PCR, and Western blot analyses, respectively. Arachidonic acid metabolites formations were determined by liquid chromatography–electron spray ionization-mass spectrometry LC-ESI-MS. Our results showed that inflammation significantly decreased the CYP epoxygenases expression in the heart, kidney and liver with a concomitant decrease in the EETs produced by these enzymes. In contrast to CYP expoxygenses, inflammation differentially altered CYP ω-hydroxylases expression with a significant increase in 20-HETE formation. The present study demonstrates for the first time that acute inflammation decreases CYP epoxygenases and their associated cardioprotective metabolites, EETs while on the other hand increases CYP ω-hydroxylases and their associated cardiotoxic metabolites, 20-HETE. These changes may be involved in the development and/or progression of cardiovascular diseases by inflammation.

1. Introduction

Recent studies have demonstrated that inflammation has a prominent role in the initiation and progression of many cardiovascular diseases [1,2]. For example, heart failure has been recognized as a complex cascade of inflammatory responses that would gradually depress cardiac functions and lead to heart failure [3]. More importantly, the role of inflammatory mediators and markers has become paramount in understanding and recognizing heart failure at earlier stages of pathogenesis. For example, high levels of tumor necrosis factor-alpha (TNF-α) and interlukin-6 (IL-6) have been serving as prognostic markers for heart failure [4,5]. Thus, it has become apparent that inflammation is a predisposing factor in heart failure, and it is also a central player in cardiac preconditioning.

Across species, inflammation causes changes in the activities and expression levels of various cytochrome P450s (CYPs) in the liver and extrahepatic tissues such as brain and kidneys [6]. In most cases, CYPs are mainly suppressed, yet some CYPs are unaffected and others are induced in response to inflammatory stimulus [6]. Moreover, CYP catalyzed metabolism of arachidonic acid has recently grabbed a lot of attention due to the fact that these metabolites are important mediators of numerous biological effects [7]. It has been well documented that arachidonic acid is metabolized by cyclooxygenase, lipooxygenase, and CYP monooxygenase pathways [8]. In addition, the CYP pathway is further divided into CYP epoxygenases and CYP ω-hydroxylases sub-pathways [8]. CYP epoxygenases are capable of producing the cardioprotective metabolites epoxyeicosatrienoic acids (EETs), which consist of four regioisomers (5,6-, 8,9-, 11,12-, 14,15-EET) [8,9]. Furthermore, CYP epoxygenases-produced EETs are rapidly degraded to the biologically less active metabolites dihydroxyeicosatrienoic acids (DHETs) via soluble epoxide hydrolase (sEH) [8]. On the other hand, CYP ω-hydroxylases produce the cardiotoxic metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) [9,10].

Early studies indicated that EETs produce important biological effects in the cardiovascular system such as regulating vascular tone in the coronary, cerebral, mesenteric, renal, pulmonary, and peripheral circulations [7,10,11]. EETs also exhibit

Abbreviations: CYP, cytochrome P450; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; IL-6, interlukin-6; IP, intraperitoneal; LC–ESI-MS, liquid chromatography–electron spray ionization-mass spectrometry; LPS, lipopolysaccharide; PCR, polymerase chain reaction.

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Table 1
Primers sequences used for real time-PCR reactions.

Gene	Forward primer	Reverse primer
β-Actin	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
CYP2B1	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGATC
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP2E1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
CYP2J3	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
CYP4A1	TTGAGCTACTGCCAGATCCCAC	CCCATTTTTGGACTTCAGCACA
CYP4A3	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATC
CYP4F1	CCCCCAAGGCTTTTTGATG	GAGCGCAACGGCAGCT
CYP4F4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
CYP4F5	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
IL-6	ATATGTTCTCAGGGAGATCTTGGAA	GTGCATCATCGCTGTTCATACA
TNF-α	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

anti-inflammatory activities [12], which in combination with their cardioprotective effects, have made EETs an attractive tool for the treatment of inflammatory-mediated cardiovascular diseases, such as cardiac hypertrophy and hear failure [13]. In contrast to EETs, 20-HETE is a potent vasoconstrictor that is produced in vascular smooth muscle cells [14]. Importantly, 20-HETE has been now implicated in many cardiovascular diseases including cardiac hypertrophy and heart failure [15–17]. For example, 20-HETE has been shown to be elevated in heart microsomes of hypertrophied and diabetic rats, suggesting more direct role of 20-HETE in cardiac preconditioning and heart failure [11,18].

Lipopolysaccharide (LPS) is an inflammatory stimulus that produces its effect largely on pivotal organs such as the liver, kidney, spleen, lung, and heart [19,20]. It has been previously demonstrated that arachidonic acid metabolism is altered during inflammation in blood vessels [20]. However, to the best of our knowledge, there has been no previous attempt to examine the effect of inflammation on cardiac arachidonic acid metabolism. Therefore, we hypothesize that alteration in cardiac arachidonic acid metabolism is due to a differential alteration in CYP epoxygnases and CYP ω-hydroxylases during inflammation. Therefore, the present study aims to address the possible effects of acute inflammation on the regulation of CYP epoxygenases and CYP ω-hydroxylases in the heart, kidney, and liver and the cardiac arachidonic acid metabolism.

2. Methods

2.1. Animals and treatment

All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague–Dawley rats weighing 300–350 g were obtained from Charles River Canada (St. Constant, QC, Canada). Animals were treated intraperitoneally (IP) with 1 mg/kg LPS (dissolved in normal saline) for 6, 12, or 24 h ($n = 6$). Weight-matched controls received the same volume of normal saline for the indicated time points. Animals at the designated time points were then euthanized under isoflurane anesthesia. All animals were allowed free access to food and water throughout the treatment period. Heart, kidney, and liver tissues were excised, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. Chemical and reagents

TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, and SYBR Green SuperMix were purchased from Applied Biosystems (Foster City, CA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (San Diego, CA) according to previously published sequences. Arachidonic acid, LPS (0127:B8), and 4-hydroxybenzophenone were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Arachidonic acid metabolite standards 5,6-, 8,9-, 11,12-, 14,15-EET, 5,6-, 8,9-, 11,12-, 14,15-DHET and 20-HETE were obtained from Cayman Chemical (Ann Arbor, MI). Reagents used for liquid chromatography–electron spray ionization-mass spectrometry (LC–ESI-MS) were at HPLC-grade. Acetonitrile and water (HPLC-grade) were purchased from EM Scientific (Gibbstawn, NJ). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

2.3. RNA extraction and cDNA synthesis

Total RNA from the frozen tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio. Thereafter, first-strand cDNA synthesis was performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μL 10× RT buffer, 0.8 μL 25× dNTP mix (100 mM), 2.0 μL 10× RT random primers, 1.0 μL MultiScribe™ reverse transcriptase, and 3.2 μL nuclease-free water. The final reaction mix was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C.

2.4. Quantification by real time-PCR

Quantitative analysis of specific mRNA expression was performed by real time-PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). 25 μL reaction mix contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. The primers used in the current study were chosen from previously published studies [21–26] and are listed in Table 1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.5. Real time-PCR data analysis

The real time-PCR data were analyzed using the relative gene expression i.e. ($\Delta\Delta$CT) method as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen [27]. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (*β-Actin*) and relative to a calibrator. The untreated control was used as the calibrator when the change of gene expression by LPS is being studied.

2.6. Microsomal protein preparation and Western blot analysis

Microsomal protein was prepared from the heart tissue as described previously [28]. Briefly, hearts were washed in ice-cold KCL (1.15%, w/v), cut into pieces, and homogenized separately in cold sucrose solution (1 g of tissue in 5 mL of 0.25 M sucrose). Microsomal protein from homogenized tissues was separated by differential ultracentrifugation. The final pellet was reconstituted in cold sucrose and stored at −80 °C. Heart microsomal protein concentration was determined by the Lowry method using bovine serum albumin as a standard [29]. Western blot analysis was performed using a previously described method [30]. Briefly, 10 or 20 μg of heart microsomal protein from each treatment group was separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween-20. After blocking, the blots were incubated with a primary polyclonal rabbit anti-rat CYP2C11, rabbit anti-rat CYP2E1, mouse anti-rat CYP4A, and rabbit anti-rat actin for 2 h at room temperature. Incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for CYP2C11, CYP2E1, and actin, or goat anti-mouse IgG secondary antibody for CYP4A was carried out for 2 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). The intensity of the protein bands was quantified, relative to the signals obtained for actin, using ImageJ software [National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij].

2.7. Microsomal incubation and separation of different arachidonic acid metabolites by LC–ESI-MS

Heart microsomes (1 mg protein/mL) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH = 7.4) at 37 °C in a shaking water bath (50 rpm). A pre-equilibration period of 5 min was performed. The reaction was initiated by the addition of 1 mM NADPH. Arachidonic acid was added to a final concentration of 50 μM and incubated for 30 min. The reaction was terminated by the addition of 600 μL ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. Arachidonic acid metabolites were extracted twice by 1 mL ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY). Extracted arachidonic acid metabolites were analyzed using LC–ESI-MS (Waters Micromass ZQ 4000 spectrometer) method as described previously [31]. The mass spectrometer was operated in negative ionization mode with single ion recorder acquisition. The nebulizer gas was obtained from an in house high purity nitrogen source. The temperature of the source was set at 150 °C, and the voltages of the capillary and the cone were 3.51 kV and 25 V, respectively. The samples (10 μL) were separated on reverse phase C18 column (Kromasil, 250 mm × 3.2 mm) using linear gradient mobile phase system water/acetonitrile with 0.005% acetic acid as mobile phase at flow rate of 0.2 mL/min. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 min, increased to 100% acetonitrile in 5 min, and held for 5 min. 4-Hydroxybenzophenone was used as internal standard.

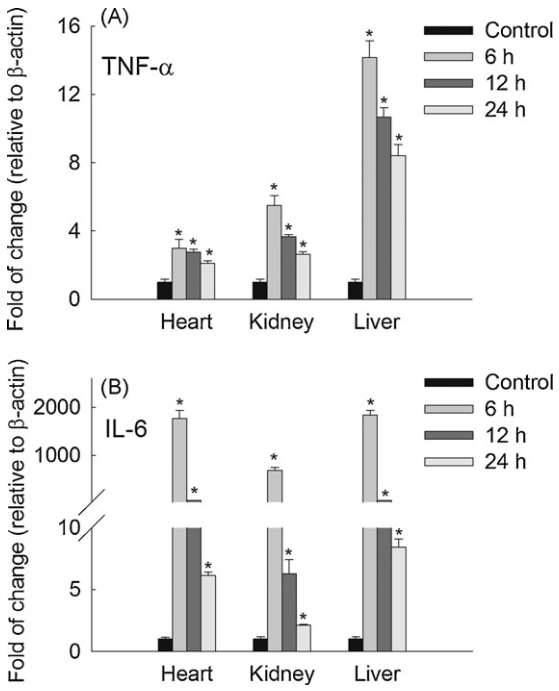

Fig. 1. Effect of LPS treatment on the inflammatory markers *TNF-α* and *IL-6* gene expression in the heart, kidney, and liver. Total RNA was isolated from the heart, kidney, and liver of control and LPS-treated animals at 6, 12, and 24 h after LPS injection. TNF-α (A) and IL-6 (B) gene expression was determined by real time-PCR. Results are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with control.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean. Comparative gene expression across different time points, and metabolites formation was analyzed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc comparison. A result was considered statistically significant where $P < 0.05$.

3. Results

3.1. Effect of LPS treatment on the inflammatory markers TNF-α and IL-6 gene expression

The dose of LPS (1 mg/kg IP) was chosen based on previously published data demonstrating that this dose produces acute systemic inflammation [32]. All animals administered LPS survived the treatment over the time course of 24 h. To confirm the induction of acute inflammatory response by LPS, total RNA was extracted from the heart, kidney, and liver of both control and LPS-treated animals at different time points. Thereafter, the expression of TNF-α and IL-6 was measured using reverse transcription followed by real time-PCR as described under Section 2. LPS treatment significantly increased TNF-α and IL-6 mRNA levels in all tissues tested at all time points (Fig. 1A and B). In the heart, TNF-α mRNA level was increased at 6, 12, and 24 h by 200%, 170%, and 110%, respectively. In addition, the kidney TNF-α mRNA level was increased at 6, 12, and 24 h by 450%, 260%, and 160%, respectively. Furthermore, liver TNF-α mRNA level was increased at 6, 12, and 24 h by 1400%, 900%, and 700%, respectively.

Similarly, IL-6 mRNA level was also increased in the heart of inflamed animals after 6, 12, and 24 h by 176,000%, 6000%, and 500%, respectively. Kidney IL-6 mRNA level at 6, 12, and 24 h was increased by 68,000%, 500%, and 100%, respectively. Lastly, liver IL-6 mRNA level at 6, 12, and 24 h was increased by 184,000%, 6000%, and 750%, respectively.

3.2. Effect of LPS treatment on CYPs gene expression

In order to investigate the effects of acute systemic inflammation on the expression of CYP genes, total RNA was extracted from liver, heart, and kidney of both control and LPS-treated rats at different time points. Thereafter, the expression of different genes was measured using reverse transcription followed by real time-PCR as described under Section 2.

Our results demonstrated that CYP1A1 mRNA levels were decreased in a time-dependent manner in all tissues tested with the heart being the most affected organ followed by the kidney and the liver. CYP1A1 mRNA levels in the heart of inflamed animals were decreased at 6, 12, and 24 h by 80%, 94%, and 96%, respectively, compared to control. Similarly, in the kidney of inflamed animals CYP1A1 mRNA level was decreased at 6, 12, and 24 h by 60%, 85%, and 92%, respectively, compared to control. Finally in the liver of inflamed animals CYP1A1 mRNA level was decreased at 6, 12, and 24 h by 45%, 89%, and 74%, respectively, compared to control (Fig. 2A). In contrast to CYP1A1, CYP1B1 mRNA level was significantly decreased in the heart and kidney, but not the liver of inflamed animals at the time point of 6 h. In the heart of inflamed animals, CYP1B1 mRNA level at 6 and 12 h was decreased by 82% and 37%, respectively, while at 24 h CYP1B1 mRNA level was increased by 80%, compared to control (Fig. 2B). Similarly in the kidney of inflamed animals, CYP1B1 mRNA level at 6 and 12 h was decreased by 77% and 36%, respectively, while at 24 h CYP1B1 mRNA level was increased by 40%, compared to control (Fig. 2B). On the other hand, in the liver of inflamed animals CYP1B1 mRNA level was increased at 6, 12, and 24 h by 76%, 150%, and 200%, respectively, compared to control (Fig. 2B).

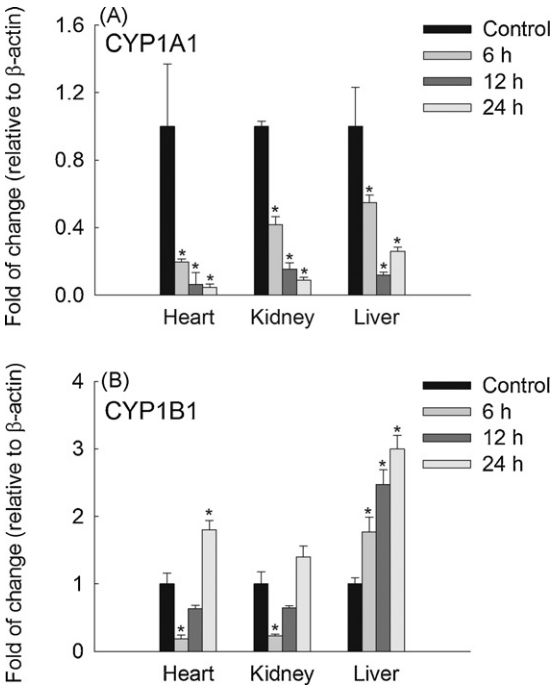

Fig. 2. Modulation of *CYP1A1* and *CYP1B1* gene expression in different tissues during LPS-induced inflammation. Inflammation was induced by a single IP injection of LPS 1 mg/kg. After 6, 12, or 24 h, the heart, kidney, and liver were collected. Total RNA was isolated and gene expression of CYP1A1 (A) and CYP1B1 (B) was determined by real time-PCR in control and LPS-treated rats. Results are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with corresponding tissue control.

With regard to the effect of inflammation on CYP2 family, CYP2B1 mRNA level was not altered in the heart of inflamed animals. On the other hand, CYP2B1 mRNA level was significantly

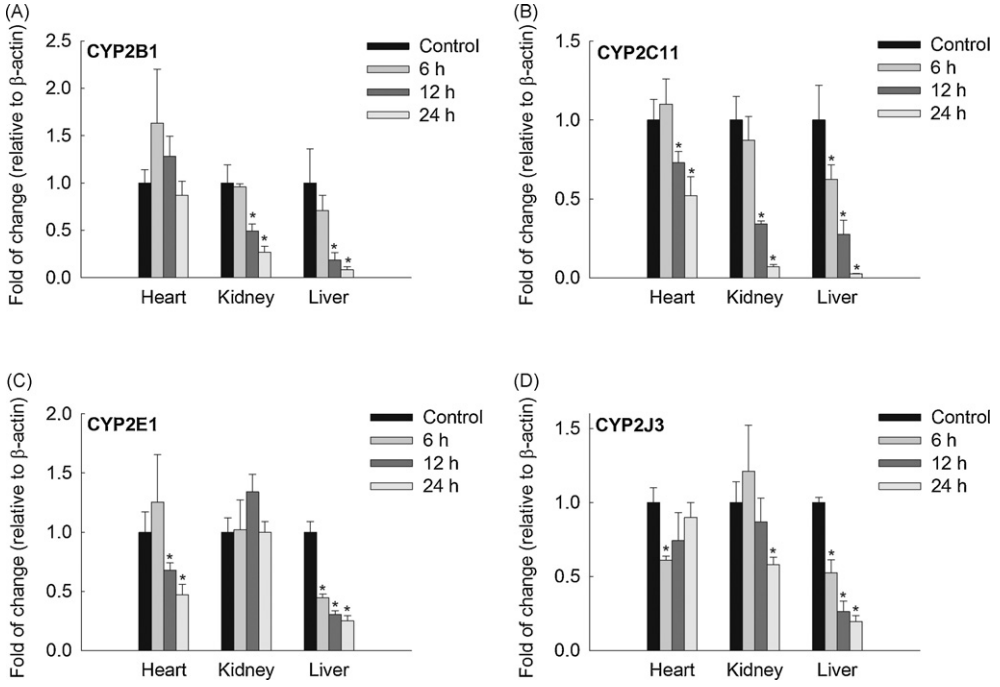

Fig. 3. Modulation of *CYP2B1*, *CYP2C11*, *CYP2E1*, and *CYP2J3* gene expression in different tissues during LPS-induced inflammation. Inflammation was induced by a single IP injection of LPS 1 mg/kg. After 6, 12, or 24 h, the heart, kidney, and liver were collected. Total RNA was isolated and gene expression of CYP2B1 (A), CYP2C11 (B), CYP2E1 (C), and CYP2J3 (D) was determined by real time-PCR in control and LPS-treated rats. Results are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with corresponding tissue control.

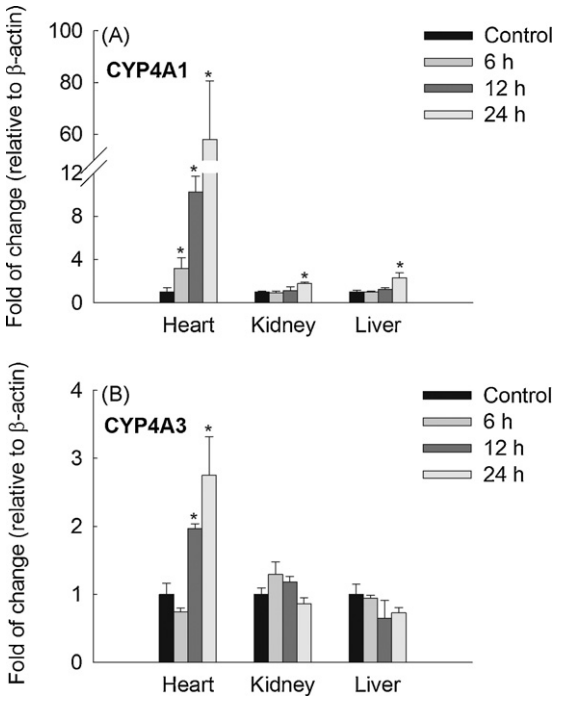

Fig. 4. Modulation of *CYP4A1* and *CYP4A3* gene expression in different tissues during LPS-induced inflammation. Inflammation was induced by a single IP injection of LPS 1 mg/kg. After 6, 12, or 24 h, the heart, kidney, and liver were collected. Total RNA was isolated and gene expression of CYP4A1 (A), and CYP4A3 (B) was determined by real time-PCR in control and LPS-treated rats. Results are presented as mean ± SEM ($n = 6$). $^*P < 0.05$ compared with corresponding tissue control.

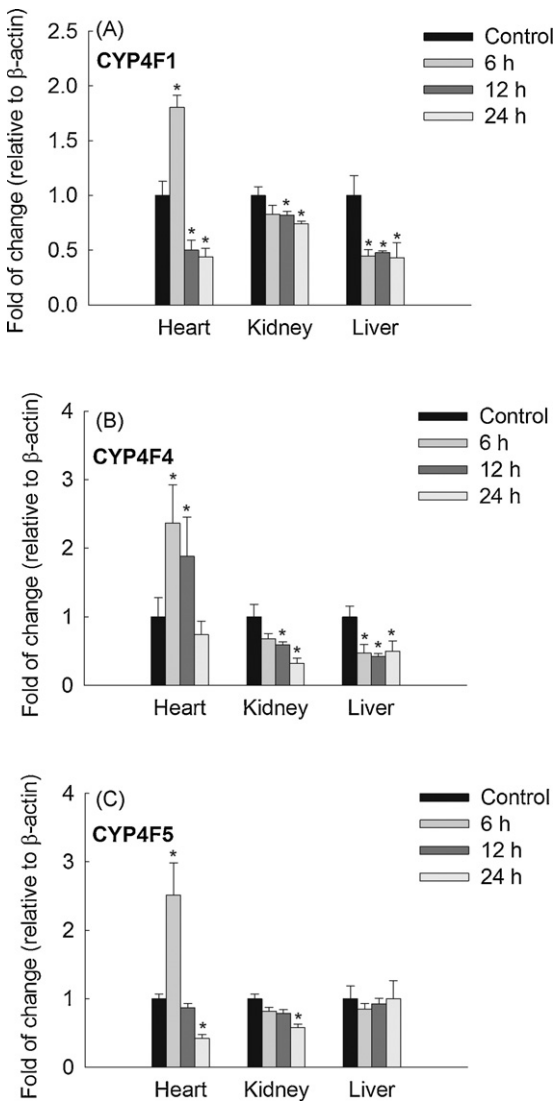

Fig. 5. Modulation of *CYP4F1*, *CYP4F4*, and *CYP4F5* gene expression in different tissues during LPS-induced inflammation. Inflammation was induced by a single IP injection of LPS 1 mg/kg. After 6, 12, or 24 h, the heart, kidney, and liver were collected. Total RNA was isolated and gene expression of CYP4F1 (A), CYP4F4 (B), and CYP4F5 (C) was determined by real time-PCR in control and LPS-treated rats. Results are presented as mean ± SEM ($n = 6$). $^*P < 0.05$ compared with corresponding tissue control.

decreased at 12 and 24 h by 50% and 70%, respectively, in the kidney and by 80% and 95%, respectively, in the liver of inflamed animals compared to control (Fig. 3A). On the other hand, CYP2C11 mRNA was significantly decreased in the heart, kidney, and liver in a time-dependent manner. The order of inhibition from highest to lowest was liver > kidney > heart. CYP2C11 mRNA level was decreased at 12 and 24 h by 25% and 50%, respectively, in the heart and by 65% and 93%, respectively, in the kidney of inflamed animals compared to control. Similarly, liver CYP2C11 mRNA level was decreased at 6, 12, and 24 h by 38%, 73%, and 98%, respectively, compared to control (Fig. 3B).

Despite of being not affected by LPS treatment in the kidney, CYP2E1 was inhibited in response to LPS treatment in the heart, and liver of inflamed animals in a time-dependent manner (Fig. 3C). In the heart of inflamed animals CYP2E1 mRNA level was decreased at 12 and 24 h by 32% and 53%, respectively, whereas in the liver it was decreased by 70 and 75%, respectively, compared to control (Fig. 3C). With regard to *CYP2J3* gene expression, there was a statistically significant decrease in CYP2J3 mRNA levels in the heart, kidney, and liver of inflamed animals that was time-dependent in the kidney and liver of inflamed animals but not in the heart. In the heart of inflamed animals CYP2J3 mRNA level was significantly decreased only at 6 h by 40% whereas in the kidney it was decreased at 24 h by 42%, compared to control. On the other hand, in the liver of inflamed animals CYP2J3 mRNA level was decreased at 6, 12, and 24 h by 48%, 74%, and 81%, respectively, compared to control (Fig. 3D).

With regard to the gene expression of the major ω-hydroxylases, Figs. 4 and 5 show the effect of inflammation on CYP4 family gene expression. Our results showed that CYP4A1 mRNA level was increased in the heart of inflamed animals in a time-dependent manner. CYP4A1 was increased in the heart of inflamed animals at 6, 12, and 24 h by 400%, 900% and 6000%, respectively, compared to control. Similarly but with a lower magnitude, kidney and liver CYP4A1 mRNA levels were increased only at 24 h in response to LPS treatment by 100% and 200%, respectively (Fig. 4A). Moreover, CYP4A3 mRNA level was increased only in the heart of inflamed animals at 12 and 24 h by 100% and 200%, respectively, compared to control (Fig. 4B). On the other hand, kidney and liver CYP4A3 mRNA levels were not altered in response to LPS treatment.

In contrast to CYP4A subfamily, all heart CYP4F mRNA levels were increased at 6 h post-inflammation and decreased at 12 and 24 h with the exception of CYP4F4 which increased at 12 h. In the heart of inflamed animals CYP4F1 mRNA level was increased at 6 h by 80% and decreased at 12 and 24 h by 50% and 66%, respectively, compared to control (Fig. 5A). In the kidney and liver of inflamed animals there was a time-dependent decrease in CYP4F1 mRNA levels. CYP4F4 mRNA level was increased in the heart of inflamed animals at 6 and 12 h by 130% and 80%, respectively, and was not altered at 24 h (Fig. 5B). In the kidney and liver of inflamed animals CYP4F4 was decreased in a time-dependent manner. Furthermore, CYP4F5 was significantly increased by 150% in the heart of inflamed animals at 6 h, while

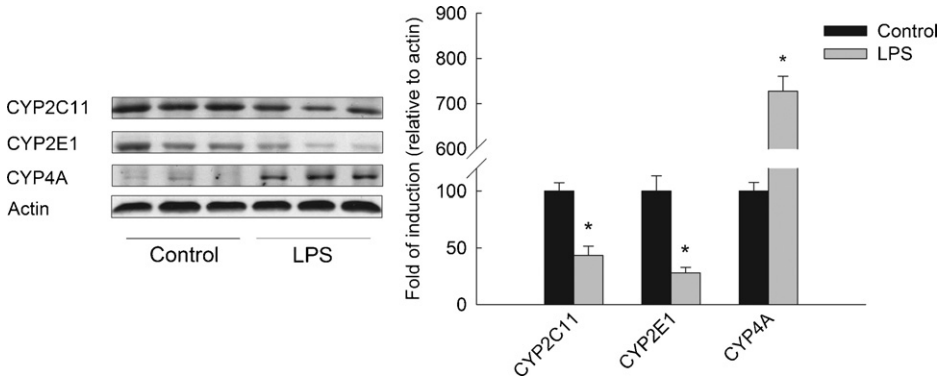

Fig. 6. Modulation of CYP2C11, CYP2E1, and CYP4A protein expression in the heart during LPS-induced inflammation. Heart microsomal protein was isolated from the heart of control and animals treated with LPS for 24 h. 10 μg of microsomal protein for CYP2C11 and CYP2E1 or 20 μg for CYP4A and actin was separated on a 10% SDS-PAGE. CYP2C11, CYP2E1, and CYP4A proteins were detected using the enhanced chemiluminescence method. The graph represents the relative amount of CYP protein normalized to the endogenous control (mean ± SEM, $n = 3$), and the results are expressed as percentage of the control values taken as 100%. *$P < 0.05$ compared with control.

it was decreased by 60% after 24 h. In the kidney of inflamed animals CYP4F5 was decreased at 24 h by 40% compared to control (Fig. 5C).

3.3. Effect of LPS treatment on CYP protein expression

To investigate whether the effect of LPS treatment on *CYP* gene expression was further translated to the protein level, microsomal protein was prepared from hearts of control and rats treated with LPS for 24 h. Thereafter, CYP2C11, CYP2E1, and CYP4A protein levels were determined using Western blot analysis relative to actin as an endogenous control. Our results show that LPS treatment significantly decreased CYP2C11 and CYP2E1 by 60% and 70%, respectively, compared to control (Fig. 6). On the other hand, LPS treatment caused a significant increase of CYP4A protein expression by 600% (Fig. 6).

3.4. Effect of LPS treatment on CYP-mediated arachidonic acid metabolism

Our results showed that EETs formation is decreased in the heart microsomes of inflamed animals. 5,6-, 8,9-, and 11,12-EETs were significantly decreased by 50%, 25%, and 35%, respectively (Fig. 7A). Of interest, the formation of 14,15-EET in heart microsomes of inflamed animals was decreased in response to LPS treatment; however, this decrease did not reach statistical significance.

To determine whether the decrease in CYP epoxygenase expression and subsequently EETs formation is reflected on the DHETs metabolites formation, we determined DHETs formation in heart microsomes from control and inflamed animals. Our results showed that 5,6-, 8,9-, 11,12-, 14,15-DHETs formations were significantly decreased in heart microsomes of inflamed animals by, 22%, 19%, 18%, and 15%, respectively (Fig. 7B). Because both EETs and DHETs are collectively epoxygenase-mediated arachidonic acid metabolites, it was of importance to measure the total EETs plus DHETs as a measurement of total epoxygenase activity. Our results showed that CYP epoxygenases activity was decreased by 30% in response to inflammation compared to control animals (Fig. 8A).

In contrast to EETs and their corresponding DHETs, inflammation significantly increased the production of 20-HETE formation in the heart microsomes of inflamed animals by 40% (Fig. 8B). Moreover, to determine the relative formation of 20-HETE compared to total EETs in the heart microsomal fraction of LPS-treated rats, we calculated the 20-HETE:total EET ratio. As shown in Fig. 8C, inflammation significantly increased the 20-HETE:EET ratio by 160%.

4. Discussion

Up to date the relationship between inflammation and arachidonic acid metabolism has been limited to the effects of EETs or 20-HETE on inflammation-mediated effect on vasculature [20,33]. Previous studies have demonstrated that 11,12-EET exerts anti-inflammatory effects on the endothelium through inhibiting the cytokine-mediated activation of the nuclear factor-κB (NF-κB) [34]. To the best of our knowledge, the effect of inflammation on CYP-mediated cardiac arachidonic acid metabolism has never been investigated previously. Therefore, in the current study we shed

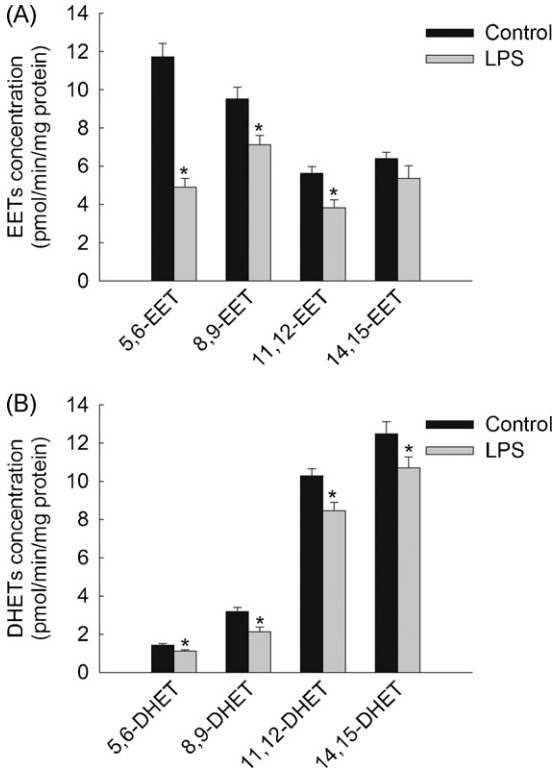

Fig. 7. Effect of LPS-induced inflammation on (A) EETs, (B) DHETs. Heart microsomes of control or LPS-treated rats for 24 h were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs and DHETs were extracted twice by 1 mL ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC–ESI-MS for metabolite determination. Results are presented as mean ± SEM ($n = 6$). *$P < 0.05$ compared with control.

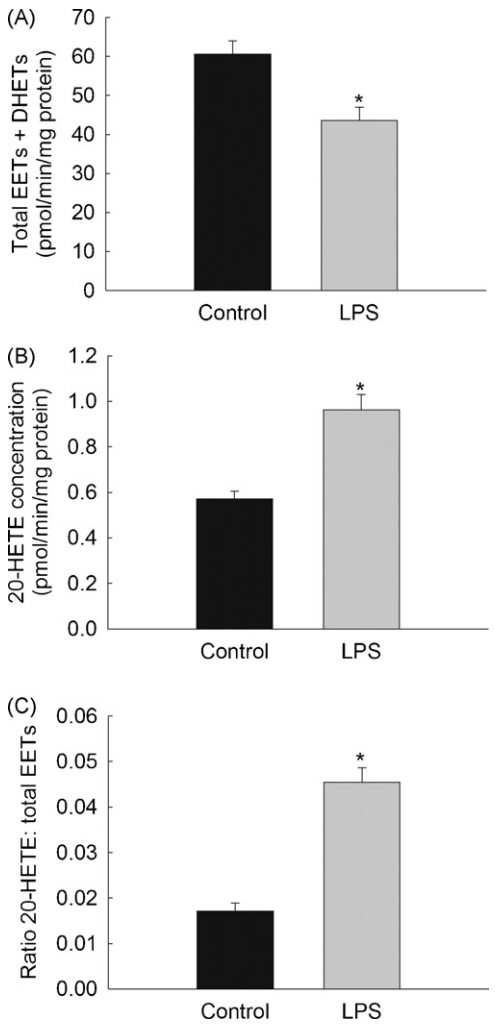

Fig. 8. Effect of LPS-induced inflammation on (A) total CYP epoxygenases activity (EETs + DHETs), (B) 20-HETE formation, and (C) total 20-HETE:EET ratio. Heart microsomes of control or LPS-treated rats for 24 h were incubated with 50 μM arachidonic acid. The reaction was started by the addition of 1 mM NADPH and lasted for 30 min. The reaction was terminated by the addition of ice-cold acetonitrile. EETs, 20-HETE, and DHETs were extracted twice by 1 mL ethyl acetate and dried using speed vacuum. Reconstituted metabolites were injected into LC–ESI-MS for metabolite determination. Results are presented as mean ± SEM ($n = 6$). $^*P < 0.05$ compared with control.

the light on the effect of inflammation on *CYPs* expression, and their associated cardiac arachidonic acid metabolism.

As a first step to confirm the inflammatory response in LPS-treated rats, we measured the inflammatory markers TNF-α and IL-6 mRNA levels. Our results showed that both biomarkers were significantly increased in response to LPS treatment. These results are in agreement with previous studies showing that TNF-α and IL-6 mRNA levels are increased in response to LPS treatment [35,36]. The magnitude of induction at different time points was 6 > 12 > 24 h. The kinetics of TNF-α and IL-6 mRNA following LPS-induced inflammation are in agreement with previous studies [37–39]. It was reported that LPS administration in mice causes a significant induction of TNF-α, and IL-6 mRNA levels with maximum induction at 3–4 h followed by rapid decline at later time points [38]. In another study it was reported that IL-6 mRNA reached its maximum induction in the kidney at 6 h following LPS administration to rats [40].

In the current study CYP1A1 was significantly decreased in all tissue of LPS-treated rats. In this regard, it has been previously demonstrated that inflammation decreases CYP1A1 mRNA levels probably due to the activation of NF-κB [41–43]. On the contrary to CYP1A1, CYP1B1 mRNA levels were selectively increased by inflammation in the liver and heart, which is in agreement with previous studies that observed similar effects in other inflammation models [44]. It has been previously reported that there are at least two regulatory pathways that control rat *CYP1B1* gene expression. The first is the hormonal regulation which maintains the constitutive expression of CYP1B1 in steroidal tissues [45], and the second is the aryl hydrocarbon receptor (AhR)-dependent pathway which governs the *CYP1B1* gene expression in response to environmental pollutants [45]. In this context, it was postulated that inflammation enhances the expression of *CYP1B1* through the hormonal pathway [44,46]. Therefore, we hypothesize that inflammation decreases CYP1B1 mRNA levels at the 6 h time point probably through inhibiting the AhR pathway, after which it increases its gene expression through affecting its hormonal pathway.

In the current study CYP2B1 mRNA level was significantly decreased in the kidney and liver but not in the heart of LPS-treated rats. Our results are in concordance with other studies showing that phenobarbital-induced CYP2B1 mRNA is rapidly decreased following LPS treatment [47]. This was explained by a significant decrease in the transcription factor that regulates CYP2B1 expression which is the constitutive androstane receptor (CAR) during inflammation [36,47,48]. With regard to CYP2C11 and CYP2E1 mRNA levels, our results showed that CYP2C11 was significantly inhibited in all tissues tested while CYP2E1 was significantly inhibited in the liver and heart but not in the kidney of LPS-treated rats. In an attempt to correlate the observed effects of LPS treatment on gene expression, we examined the effect of LPS treatment on CYP2C11 and CYP2E1 protein expression. In agreement with CYP gene expression data CYP2C11 and CYP2E1 protein expression levels were decreased by 60% and 70%, respectively. Our results are in agreement with previously published studies showing that inflammation causes down-regulation of these enzymes upon treatment by LPS [49]. Of interest, CYP2J3 mRNA level was also decreased in the heart, kidney, and the liver of LPS-treated rats. To the best of our knowledge, our results show for the first time that inflammation decreases the constitutive expression of CYP2J3 mRNA levels in the heart, kidney, and the liver.

The role of the previously mentioned CYP enzymes in arachidonic acid metabolism has been previously demonstrated. For example, CYP1A1 has been shown to be involved in ω-terminal HETE synthesis, whereas CYP1B1 can metabolize arachidonic acid to both mid-chain HETEs and EETs [50]. CYP2B1, CYP2C11, CYP2E1, and CYP2J3 are major epoxygenase enzymes that are involved in arachidonic acid metabolism to EETs [13,51,52]. In addition, CYP2E1 has been reported to metabolize arachidonic acid to 18- and 19-HETEs [53]. The fact that inflammation decreased CYP epoxygenases gene expression typified by CYP2B1, CYP2C11, and CYP2J3 prompted us to examine the effect of inflammation on the CYP ω-hydroxylases gene expression. Our results demonstrated that the gene expression of *CYP4A1* is increased in all tissues tested, and *CYP4A3* was only increased in the heart of LPS-treated animals. In accordance with the CYP expression data, there was a significant induction of CYP4A protein by 600% in the heart microsomes of rats treated with LPS for 24 h. These results are in agreement with previous studies demonstrating that inflammation increases hepatic CYP4A1 and CYP4A3 mRNA levels in LPS-treated rats [32]. However, in this study we showed for the first time that CYP4A1 and CYP4A3 gene expression is increased in hearts of acutely inflamed animals.

The gene expression of CYP4F genes was altered by LPS treatment in a tissue-specific manner. First, CYP4F1 mRNA level was significantly increased in the heart at the early time point and decreased at later time points similar to what is observed in the

kidney and liver of LPS-treated rats. Second, CYP4F4 mRNA level was significantly increased in the heart and decreased in the liver and kidney of LPS-treated rats. Third, CYP4F5 mRNA level was similar to CYP4F1 in that its mRNA level was initially increased at early time point and then significantly decreased at the later time points in the heart of LPS-treated rats whereas it decreased in the kidney but not in the liver of LPS-treated rats. In agreement with our studies, liver CYP4F4 was decreased in response to LPS treatment in Fisher rats [54]. In addition, *in vitro* studies using isolated primary rat hepatocytes previously showed that LPS treatment causes a general decrease in all CYP4F subfamily mRNA levels [55]. However, in contrast to our results, it has been previously reported that liver CYP4F1 and CYP4F6 were not affected, while CYP4F5 was induced by LPS treatment in Fisher rats [54]. Thus, the contradiction between our results and previously published studies could be attributed to differences in rat strain or time points which might reflect the differences in the responses to LPS treatment.

In an attempt to correlate the effects of inflammation on CYP epoxygenases typified by CYP2B1, CYP2E1, CYP2C11, and CYP2J3 to EETs formation, we determined EETs formation in the heart microsomes from control and LPS-treated rats. Our results demonstrated for the first time that all EET regioisomers formation with the exception of 14,15-EET was significantly decreased in the heart microsomal fractions of LPS-treated rats. In addition, the total heart EETs plus DHETs metabolites formation which reflects the total epoxygenase activity was significantly decreased in response to LPS treatment. These results matched the gene and protein expression data in which CYP epoxygenases were significantly decreased in the heart of LPS-treated rats. Thus, it is apparent that inflammation through decreasing the expression of CYP epoxygenases causes a decrease in the cardioprotective metabolites, EETs.

To determine whether the increase in CYP ω-hydroxylases gene expression led to alteration in 20-HETE formation, we determined 20-HETE formation in the heart microsomal fractions from control and LPS-treated rats. Our results showed for the first time that inflammation causes an increase in the production of 20-HETE in the heart microsomal fractions of LPS-treated rats. Therefore, inflammation via increasing CYP1B1, CYP4A1, CYP4A3, and CYP4F contributed to the increase in the production of 20-HETE in inflamed animals heart microsomes [8,50]. The increase in the 20-HETE production could be a compensatory mechanism by which the inflamed hearts react to prevent a systemic hypotensive shock [20]. In the current study, the 20-HETE:total EETs ratio, was significantly higher in LPS-treated rats compared to control. Therefore, acute inflammation causes alteration in cardiac CYP-mediated arachidonic acid metabolism in favor of 20-HETE formation. Interestingly, inhibition of 20-HETE formation caused improvement of the cardiac function following ischemia-reperfusion injury in diabetic rats [56] and reduced cardiomyocyte apoptosis in another model of ischemia-reperfusion injury [16]. Taken together, CYP ω-hydroxylase inhibitors may provide a future approach for the protection against inflammation-mediated heart diseases.

In conclusion, the present study demonstrates for the first time that inflammation might participate in pathogenesis of cardiovascular diseases through altering CYPs and their associated cardiac arachidonic acid metabolism. The overall effect of inflammation on arachidonic acid metabolism was a decrease in the cardioprotective metabolite, EETs and an increase in the cardiotoxic metabolite, 20-HETE. These changes may be involved, at least in part, in the development and/or progression of cardiovascular diseases. In addition, reversal of these changes by increasing the EETs or inhibiting the 20-HETE formation may provide new strategies for the prevention and treatment of inflammation-mediated cardiovascular diseases.

Conflict of interests

The authors declare no conflicts of interests.

Acknowledgments

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