Arachidonic acid stimulates TNFα production in Kupffer cells via a reactive oxygen species-pERK1/2-Egr1-dependent mechanism

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Cubero FJ, Nieto N. Arachidonic acid stimulates TNFα production in Kupffer cells via a reactive oxygen species-pERK1/2-Egr1-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 303: G228–G239, 2012. First published April 26, 2012; doi:10.1152/ajpgi.00465.2011.—Kupffer cells are a key source of mediators of alcohol-induced liver damage such as reactive oxygen species, chemokines, growth factors, and eicosanoids. Since diets rich in polyunsaturated fatty acids are a requirement for the development of alcoholic liver disease, we hypothesized that polyunsaturated fatty acids could synergize with ethanol to promote Kupffer cell activation and TNFα production, hence, contributing to liver injury. Primary Kupffer cells from control and from ethanol-fed rats incubated with arachidonic acid showed similar proliferation rates than nontreated controls; however, arachidonic acid induced phenotypic changes, lipid peroxidation, hydroperoxides, and superoxide radical generation. Similar effects occurred in human Kupffer cells. These events were greater in Kupffer cells from ethanol-fed rats, and antioxidants and inhibitors of arachidonic acid metabolism prevented them. Arachidonic acid treatment increased NADPH oxidase activity. Inhibitors of NADPH oxidase and of arachidonic acid metabolism partially prevented the increase in oxidant stress. Upon arachidonic acid stimulation, there was a rapid and sustained increase in TNFα, which was greater in Kupffer cells from ethanol-fed rats than in Kupffer cells from control rats. Arachidonic acid induced ERK1/2 phosphorylation and nuclear translocation of early growth response-1 (Egr1), and ethanol synergized with arachidonic acid to promote this effect. PD98059, a mitogen extracellular kinase 1/2 inhibitor, and curcumin, an Egr1 inhibitor, blocked the arachidonic acid-mediated upregulation of TNFα in Kupffer cells. This study unravels the mechanism whereby arachidonic acid and ethanol increase TNFα production in Kupffer cells, thus contributing to alcoholic liver disease.

polyunsaturated fatty acids; alcoholic liver disease; reduced nicotinamide adenine dinucleotide phosphate oxidase; oxidative stress; antioxidants; early growth response-1; tumor necrosis factor-α; extracellular signal-regulated kinase

ALCOHOLIC LIVER DISEASE (ALD) is mediated, among others, by activation of Kupffer cells (KC; Refs. 2, 12, 20, 39, 44–45, 48). It has been demonstrated that alcohol-induced liver injury occurs only in rodents fed ethanol with polyunsaturated (PUFAs) but not with saturated fatty acids. Enhanced oxidative stress, circulating endotoxin, and proinflammatory chemokines also play a major role in the pathogenesis of ALD (24–25). In addition, PUFAs are precursors for the synthesis of eicosanoids and monohydroxy eicosatetraenoic acids, contributing to the inflammatory response.

KC are resident macrophages recruited upon the onset of liver injury and are known to play a central role in the homeostatic response to hepatic damage. Upon stimulation, they immediately respond to the insult and release mediators that drive inflammatory and reparative responses, and they defend against invading microorganisms and function as the major site for endotoxin clearance (43). Once activated, KC release a wide array of soluble mediators such as reactive oxygen species (ROS), nitric oxide, chemokines, growth factors, cyclooxygenase (COX), and lipoxygenase (LOX) metabolites, all of which provide pivotal paracrine effects on other liver cells (10, 27, 43).

In ALD, the cascade of events activating KC initiates in response to enhanced translocation of Gram-negative bacteria from the gut into the portal circulation, leading to increased endotoxin levels and stimulation of KC to produce TNFα and other proinflammatory mediators. TNFα is the master initiator of the hepatic inflammatory response (3, 17, 43), and it induces the expression of chemokines, facilitating the transmigration of inflammatory cells into the subendothelial space (17, 43, 45). KC-derived TNFα also affects hepatocyte viability, hence, contributing to alcohol-induced liver injury (1, 4, 35). Thus the homeostatic responses are initiated by KC-derived mediators to promote reparative mechanisms against damage (42).

A key source of ROS in KC is NADPH oxidase. During the oxidative burst of KC phagocytic activity, NADPH oxidase catalyzes a one-electron reduction of O2− to generate superoxide radical (O2·−) at the expense of NADPH. Under normal conditions, KC have limited ability to generate ROS; however, after a “priming” event, such as that mediated by endotoxin or chemokines, they increase ROS production, which becomes maximal during phagocytosis.

PUFAs are a requirement for the development of ALD, and TNFα plays a significant role in the pathogenesis of ALD. We (10) previously demonstrated that ethanol in combination with PUFAs upregulates two powerful antifibrogenic molecules such as TNFα and glutathione (GSH). In the current work, we focused on analyzing the underlying mechanisms by which PUFAs and ethanol promote KC activation and TNFα production, thus contributing to liver injury. This study identified a key role for NADPH oxidase-derived ROS, extracellular ERK1/2 phosphorylation, and early growth response-1 (Egr1) nuclear translocation to the mechanism whereby ethanol and PUFAs synergize to upregulate TNFα production in KC.

MATERIALS AND METHODS

Isolation of primary KC. Primary rat KC were isolated using pronase and liberase blendzyme-3 (Roche, Indianapolis, IN) followed by density gradient in Optiprep (11% over 17%) and elutriation.
according to previously established protocols (28). Primary human KC were isolated from normal liver margin of patients undergoing hepatic tumor resection following the same procedure albeit using higher concentration of enzymes (28). Human KC were kindly donated by Dr. Hong (Mount Sinai School of Medicine, New York, NY). Cells were further purified by cell sorting using CD163-FITC as a KC-specific marker. KC can be maintained up to 14 days in culture preserving their ultrastructural and cytochemical characteristics while becoming activated over time. KC (5 × 10⁵ cells) were seeded on sixwell plates using DMEM-F12 with 10% FBS. The medium was replaced every 2 days, 0–10 μM arachidonic acid (AA) were added 6 days later, and the cell culture medium and cell lysates were collected at selected time points, depending on the experiment, for subsequent assays.

**Chronic alcohol feeding model.** Rats (300 g female Sprague-Dawley; n = 10/group) were fed either the control or the ethanol Lieber-DeCarli diets (21) (Bio-Serv, Frenchtown, NJ) for 8 mo with progressive increase in ethanol-derived calories (1 wk with 10%, 1 wk with 20%, and 7.5 mo with 35%). Rats were pair fed throughout the experiment. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health. The protocol was approved by the IACUC office at our institution.

**General methodology.** Endotoxin-free AA, to avoid KC activation, was conjugated to BSA as previously described (10). Cell viability under each treatment was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell proliferation was calculated from the rate of incorporation of methyl[3H]thymidine into the DNA of KC (29). Secreted TNFα was measured by ELISA (Invitrogen, Carlsbad, CA) and intracellular TNFα by flow cytometry using a TNFα-PE Ab (BD Biosciences, San Diego, CA). ATP levels

<table>
<thead>
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<th>Gene</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
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<td>TGGAGACTGCTCTCCCAAGTTA</td>
</tr>
<tr>
<td>Human TNFα</td>
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<td>CCTCTGATGGACGGACGCAG</td>
</tr>
<tr>
<td>Rat GAPDH</td>
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<td>AGGATGATCTTTTGGGCTGC</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>CAATGACCCCTTGATGGACC</td>
<td>GATCTGCTCTGGATG</td>
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</table>

**Table 1. Primers for quantitative RT-PCR**

![Fig. 1. Arachidonic acid (AA) exposure increases intra- and extracellular lipid peroxidation (LPO) in Kupffer cells (KC). KC from control (KCControl) and from ethanol-fed rats (KCEthanol) and human KC were incubated with 0–10 μM AA for 24 h. Intracellular LPO measured by *cis*-parinaric acid fluorescence in KCControl and KCEthanol (A, left) and in human KC (A, right). Extracellular LPO end products analyzed by thiobarbituric acid reactive substances (TBARS) in KCControl and KCEthanol (B, left) and in human KC (B, right) were determined. KCControl and KCEthanol were also co-treated with 0–50 μM vitamin E, 0–200 U/ml catalase, or the combination of both. Results are calculated as fold change over the nontreated KCControl and are expressed as means ± SE for n = 6. AUF, arbitrary units of fluorescence. *P < 0.05, **P < 0.01, and ***P < 0.001 for AA treated vs. control; **P < 0.01 and ***P < 0.001 for antioxidant treated or co-treated vs. control; **P < 0.05 and ***P < 0.01 for KCEthanol vs. KCControl.**

```python
def calculate_fold_change(control, treated):
    return treated / control

control = 100
AA_control = calculate_fold_change(control, 120)
AA_antioxidant = calculate_fold_change(control, 150)
AA_combination = calculate_fold_change(control, 170)
```
were determined using the luciferase ATP assay kit (Sigma, St. Louis, MO).

**Oxidant stress measurements.** Intracellular lipid peroxidation (LPO) was determined by addition of 10 μM cis-parinaric acid (Invitrogen) for 1 h and measurement of the fluorescence signal at 405 nm, based on the method of Hedley and Chow (16). Thiobarbituric acid reactive substances (TBARS) were assayed to measure intracellular lipid peroxidation according to the method of Fraga et al. (13) calculating the concentration of malondialdehyde from a standard curve of malondialdehyde bis-dimethylacetal. Intracellular hydroperoxides [mostly hydrogen peroxide (H₂O₂)] and O₂⁻ were assessed by flow cytometry using the fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium, respectively (Invitrogen). Extracellular hydroperoxides (mostly H₂O₂) were measured using the ferrous oxidation-xylenol orange assay (22). GSH levels, catalase, and SOD activities were measured according to published methods (6, 9, 15, 30, 33).

**Enzymatic activities.** CYP2E1 activity was measured using the fluorescent substrate 7-methoxy-4-trifluoromethyl coumarin (Sigma) (11). Xanthine oxidase activity was calculated from the rate of formation of urate from hypoxanthine. NADPH oxidase activity was determined by the lucigenin assay as described previously (7).

### Fig. 2. AA treatment increases intra- and extracellular hydroperoxides in KC. A time-course experiment was carried out with KCControl in the presence of 0–10 μM AA from 0 to 24 h and intracellular hydroperoxides were measured by 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence (A). KCControl, KCEthanol and human KC were incubated with 0–10 μM AA for 24 h. Intracellular hydroperoxides (mostly H₂O₂) were assessed by DCFDA fluorescence in KCControl and KCEthanol (B, left) and in human KC (B, right). Hydroperoxides secreted to the culture medium were measured by the ferrous oxidation-xylenol orange method in KCControl and KCEthanol (C, left) and in human KC (C, right). KCControl and KCEthanol were also cotreated with 0–50 μM vitamin E, 0–200 U/ml catalase, or the combination of both. Results are calculated as fold change over the nontreated KCControl and are expressed as means ± SE for n = 6. *P < 0.05, **P < 0.01 and ***P < 0.001 for AA treated vs. control; *P < 0.05, **P < 0.01, and ***P < 0.001 for antioxidant treated or cotreated vs. control; *P < 0.05, **P < 0.01, and ***P < 0.001 for KCEthanol vs. KCControl.
Quantitative RT-PCR. Total RNA was extracted from KC using the PureLink micro-to-midi total RNA purification system (Invitrogen). cDNA was synthesized using the FastStart PCR Master Mix (Roche), and quantitative RT-PCR reactions were performed with the Roche Light Cycler. Primers for the Light Cycler reaction are listed on Table 1. The specific target expression was calculated using the cycle threshold CT. Quantification of the intensity of the Western blot bands was carried out using NIH ImageJ software.

Western blot analysis. Western blot analysis was performed with proteins from cell lysate or from nuclear extracts as previously described (23). Primary antibodies (1/2,000 to 1/5,000) for total ERK1/2 and pERK1/2 were purchased from Cell Signaling (Danvers, MA). Antibodies for Egr1, GAPDH, and β-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG and goat anti-mouse IgG (1/5,000; Chemicon, Billerica, MA) were used as secondary antibodies. The signal was detected using the ECL system (GE Healthcare, Princeton, NJ) using a Laser4000 (Fujitsu, Stamford, CT). Quantification of the intensity of the Western blot bands was carried out using NIH ImageJ software.

Statistical analysis. Data were analyzed by a two-factor ANOVA. Results are expressed as means ± SE (n > 6).

RESULTS

As previously reported (10), our initial experiments involved dose-response and a time-course studies to determine the dose of AA and the time of incubation of KC from control (KCControl) and from ethanol-fed rats (KCEthanol) that did not alter cell viability but caused a modest increase in cell proliferation in primary KCEthanol.

Table 2. Antioxidant defense in KCControl and KCEthanol

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 μM</th>
<th>Control</th>
<th>10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH, nmol/mg prot</td>
<td>15.7 ± 0.2</td>
<td>16.8 ± 0.5</td>
<td>23.3 ± 2.4*</td>
<td>27.9 ± 0.3***</td>
</tr>
<tr>
<td>SOD, U/mg prot</td>
<td>0.8 ± 0.2</td>
<td>1.7 ± 0.8*</td>
<td>0.8 ± 0.25</td>
<td>1.6 ± 0.6*</td>
</tr>
<tr>
<td>Catalase, U/mg prot</td>
<td>55 ± 0.8</td>
<td>133 ± 0.6***</td>
<td>60 ± 1.2*</td>
<td>144 ± 3.8***</td>
</tr>
</tbody>
</table>

Results are means ± SE; n = 6. GSH levels, SOD, and catalase activities were determined in KC from control (KCControl) and from ethanol-fed rats (KCEthanol) in the presence of 0–10 μM arachidonic acid (AA). *P < 0.05 and ***P < 0.001 for AA treated vs. control; *P < 0.05 and ***P < 0.001 for KCEthanol vs. KCControl.

Table 3. Antioxidant defense in human KC

<table>
<thead>
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<th>Human KC</th>
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<tbody>
<tr>
<td>Control</td>
<td>10 μM</td>
</tr>
<tr>
<td>GSH, nmol/mg prot</td>
<td>14.2 ± 0.8</td>
</tr>
<tr>
<td>SOD, nmol/mg prot</td>
<td>671 ± 9.4</td>
</tr>
<tr>
<td>Catalase, U/mg prot</td>
<td>48.4 ± 1.9</td>
</tr>
</tbody>
</table>

Results are means ± SE; n = 3. GSH levels, SOD, and catalase activities were measured in human KC in the presence of 0–10 μM AA. *P < 0.05 for AA treated vs. control.
AA induces oxidant stress in KC. Since the AA challenge induced phenotypic changes indicative of KC activation, we next measured the levels of candidate mediators that could affect key downstream targets in KC, such as TNFα, due to its role in ALD. Intracellular LPO end products, hydroperoxides (mostly H₂O₂), and O₂⁻ as well as extracellular TBARS and hydroperoxides (mainly H₂O₂) were measured. There was a 40% increase in intracellular LPO over KCControl and a 25% increase over KCEthanol after AA treatment (Fig. 1A, left). A twofold increase was observed in control human KC (Fig. 1A, right). Secreted LPO-derived products increased by 20% in KCControl and by 60% in KCEthanol due to AA treatment (Fig. 1B, left). Extracellular LPO was 50% higher in AA-treated control human KC than in non-treated cells (Fig. 1B, right). The effects in rat KC were blocked by either vitamin E, which prevents LPO; catalase, which decomposes H₂O₂; or by the combination of both (Fig. 1A and B, left). Thus these experiments suggest that AA induces LPO more in KCEthanol than in KCControl.

Since hydroperoxides participate in alcohol-mediated liver injury, we next determined the effects of AA on the generation of hydroperoxides. Because AA caused a rapid and sustained induction of intracellular hydroperoxides in KCControl (Fig. 2A), we then compared the AA-mediated generation of hydroperoxides in KCControl and KCEthanol at the 24-h time point. AA increased intracellular hydroperoxides generation by 100% in KCControl and by 40% in KCEthanol (Fig. 2B, left) as well as by sevenfold in control human KC (Fig. 2B, right) compared with their respective controls. Likewise, extracellular hydroperoxides increased by 50% in KCControl (Fig. 2C, left) and by 20% in control human KC (Fig. 2C, right) by AA compared with their respective controls. The induction of both intracellular and extracellular hydroperoxides in KCControl and KCEthanol in rat KC was blunted by vitamin E, by catalase, or by the combination of both (Fig. 2; B and C, left). Overall, the data indicate that AA induces the generation of hydroperoxides, mainly intracellular, more in KCEthanol than in KCControl.

Lastly, we measured the effects of AA on O₂⁻ generation, a highly reactive non-diffusible species involved in ALD. Because AA caused a rapid and sustained induction of intracellular O₂⁻ in KCControl (Fig. 3A), we then compared the effect of the AA-driven O₂⁻ generation in KCControl and KCEthanol at the 24-h time point. Intracellular levels of O₂⁻ were elevated by 60% both in KCControl and in KCEthanol (Fig. 3B, left) and by fivefold in control human KC (Fig. 3B, right) treated with AA compared with their respective controls. The effects on rat KC were prevented by addition of vitamin E, by catalase, or by the combination of both (Fig. 3B, left). In aggregate, these results demonstrate that AA elevates O₂⁻ more in KCEthanol than in KCControl.

Since AA elevated LPO and ROS more in KCEthanol than in KCControl, we next determined whether additional changes occurred in the cellular antioxidant defense by analyzing the activity of antioxidant enzymes as well as GSH levels. Both in KCControl and in human KC, GSH levels remained similar in the absence or presence of AA but increased slightly by AA challenge in KCEthanol; however, AA elevated SOD and catalase activities in all cases (Tables 2 and 3). Thus AA increased the antioxidant defense in KC, possibly as a protection to counteract the increase in prooxidant species.

We then asked whether AA could alter ATP levels and/or affect the activity of key enzymes known to generate ROS. AA slightly increased ATP levels in KCControl, KCEthanol, and slightly in control human KC (Tables 4 and 5). Xanthine oxidase and cytochrome P450 2E1 (CYP2E1) activities remained quite similar in no-treated KCControl, KCEthanol and in control human KC but were slightly elevated by AA challenge in all cases (Tables 4 and 5). As previously shown (10), NADPH oxidase activity increased by 80% in KCControl, by 140% in KCEthanol, and by 50% in control human KC treated with AA (Tables 4 and 5). Hence, induction of NADPH oxidase activity by AA challenge could condition further downstream noxious effects in KC.

To prove that AA upregulation of NADPH oxidase activity contributed to ROS generation, KCControl and KCEthanol were preincubated with diphenyleneiodonium (DPI), a NADPH oxidase inhibitor. DPI reduced the AA-mediated increase in intracellular O₂⁻ and LPO in KCControl and KCEthanol compared with nontreated cells (Table 6). TBARS and secreted hydroperoxides were also elevated (not shown). Therefore, these results prove that NADPH oxidase plays a major role in the effects mediated by AA on KC-derived ROS generation.

To determine whether the effects on KC-mediated ROS production were exclusive of AA, as a representative PUFA, or were also induced by other fatty acids, KCControl and KCEthanol were incubated with oleic acid (OA), a monounsaturated fatty acid, or with eicosapentaenoic acid (EPA), a n-3 series PUFA. Intracellular and secreted hydroperoxides (mostly H₂O₂) were elevated in the presence of AA and EPA but not by OA (Fig. 4, A and B). The effects on intracellular hydroperoxides were greater in KCEthanol. Similar results were observed for LPO and O₂⁻ (not shown). Thus PUFAs in combination with ethanol increase ROS generation by KC and this effect is higher in KCEthanol than in KCControl.

### Table 4. ATP levels and sources of ROS in KCControl and KCEthanol

<table>
<thead>
<tr>
<th>Source</th>
<th>Control 10 μM</th>
<th>KCEthanol 10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, AU/mg prot</td>
<td>18 ± 0.9</td>
<td>18.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>22 ± 1.2</td>
<td>21 ± 0.3*</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>41 ± 3.0</td>
<td>47 ± 1.7</td>
</tr>
<tr>
<td>U/mg prot</td>
<td>40 ± 1.0</td>
<td>50 ± 2.3*</td>
</tr>
<tr>
<td>CYP2E1, AU/mg prot</td>
<td>7.5 ± 0.1</td>
<td>9.7 ± 0.1*</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>13 ± 0.8</td>
<td>23.3 ± 0.3**</td>
</tr>
<tr>
<td>U/mg prot</td>
<td>22.3 ± 1.0</td>
<td>53.7 ± 1.2**</td>
</tr>
</tbody>
</table>

Results are means ± SE; n = 6. ATP levels, NADPH oxidase, xanthine oxidase, and cytochrome P450 2E1 (CYP2E1) activities were determined in KCControl and KCEthanol in the presence of 0–10 μM AA. AU, arbitrary units. *p < 0.05 and **p < 0.01 for AA treated vs. control. #p < 0.05 and †p < 0.01 for KCEthanol vs. KCControl.

### Table 5. ATP levels and sources of ROS in human KC

<table>
<thead>
<tr>
<th>Source</th>
<th>Control 10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, AU/mg prot</td>
<td>11.3 ± 1.5</td>
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<td></td>
<td>13.7 ± 1.7</td>
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<td>Xanthine oxidase</td>
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<tr>
<td>U/mg prot</td>
<td>41.1 ± 1.8</td>
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<td>CYP2E1, AU/mg prot</td>
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<td>NADPH oxidase</td>
<td>10.6 ± 0.2</td>
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<tr>
<td>U/mg prot</td>
<td>16.2 ± 0.7*</td>
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</table>

Results are means ± SE; n = 3. ATP levels, NADPH oxidase, xanthine oxidase, and CYP2E1 activities were measured in human KC in the presence of 0–10 μM AA. ROS, reactive oxygen species. *p < 0.05 and **p < 0.01 for AA treated vs. control.
Lastly, we evaluated whether AA could also enhance ROS generation by its own metabolism via the COX and/or the LOX pathways. Using NS398 and nordihydroguaiaretic acid (NDGA), inhibitors of the COX and the LOX pathways, respectively, we measured intracellular (Fig. 4C) and secreted hydroperoxides (not shown). AA elevated hydroperoxides more in KC Ethanol than in KC Control, which was blocked by NDGA and to a lesser extent by NS398, suggesting that AA metabolism via the LOX and COX pathways also contributes to enhance oxidant stress (Fig. 4C).

AA upregulates TNFα in KC. Because we hypothesized that AA could regulate ROS generation in KC and as a corollary elevate TNFα production, first, to determine the time point for maximal TNFα induction under AA challenge, we carried out a time-course experiment from 0–24 h and measured intra- and extracellular TNFα in KC Control and in KC Ethanol. AA rapidly increased intracellular TNFα protein (Fig. 5A) and mRNA (not shown), while secreted TNFα peaked at 1 h decaying thereafter in KC Control and peaked at 3 h but remained elevated over a 24 h period in KC Ethanol (Fig. 5B). Antioxidants (vitamin E, catalase or the combination of both) and NADPH oxidase inhibitors (apocynin and DPI) decreased the AA-stimulated intracellular TNFα induction (Fig. 5, C and D) and TNFα secretion (not shown). Likewise, blocking AA metabolism with NS398 or NDGA prevented the AA-mediated increase in TNFα production over 24 h both in KC Control and in KC Ethanol (Fig. 5E). Therefore, the AA-mediated ROS increase, likely via NADPH oxidase activity and AA metabolism, regulates TNFα production, which is greater in KC Ethanol than in KC Control.

**ERK1/2 phosphorylation conditions TNFα upregulation by AA in KC.** Since stress-activated kinases are sensitive to prooxidants, we tested whether these kinases could play a role in the AA induction of TNFα. Analysis of ROS-sensitive kinases showed that AA increased only pERK1/2 but not pJNK or p38. This expression was significantly higher in KC Ethanol than in KC Control (Fig. 6A). This increase was blunted to some extent by DPI—a NADPH oxidase inhibitor—and completely blocked by vitamin E (Fig. 6B). To link AA with pERK1/2 and TNFα upregulation, cells were treated with PD098059, an inhibitor of the mitogen extracellular kinase pathway. PD098059 prevented both pERK1/2 expression and TNFα upregulation by AA in KC, thus establishing a connection among AA, pERK1/2, and the TNFα increase (Fig. 6, C and D).

**Egr1 acts as a downstream signal in the ROS-pERK1/2-dependent Upregulation of TNFα by AA in KC.** Lastly, we evaluated whether Egr1, a direct target of pERK1/2, was responsible for the TNFα induction by AA in KC. Nuclear translocation of Egr1 significantly increased in KC Ethanol exposed to AA up to 1 hour compared with KC Control (Fig. 7A). Next, we studied the expression of Egr1 within the first h after treatment with AA in the presence of PD098059. Blocking pERK1/2, lowered nuclear translocation of Egr1 more in KC Ethanol than in KC Control (Fig. 7B). Finally, to determine whether a link existed between Egr1 translocation and the AA-mediated induction of TNFα, cells were coincubated with curcumin, an inhibitor or Egr1 (8, 14), along with AA, and TNFα levels and intracellular hydroperoxides were measured. Curcumin blocked the AA-mediated induction of TNFα more in KC Ethanol than in KC Control and prevented the generation of intracellular hydroperoxides (Fig. 7, C and D), hence establishing a link among AA induction of ROS, AA-increased Egr1 nuclear translocation, and TNFα induction in KC.

**DISCUSSION**

A growing body of work on ALD suggests a two-hit theory where fat and alcohol work in a consorted fashion to cause liver injury. While dietary fat contributes to the development of alcohol-induced liver damage, the source of fat is fundamental (26, 40). Indeed, diets containing PUFAs promote, whereas diets containing saturated fat prevent liver injury (24). We (10) previously demonstrated that chronic ethanol feeding and a representative PUFA (e.g., AA) modulate KC activation and the fibrogenic response in HSC in coculture with KC. The present study focused on elucidating the mechanism whereby AA and ethanol synergize to upregulate TNFα production in KC, thus contributing to the pathophysiology of ALD.

AA activated KC as depicted by increased cytoplasmic processes and membrane ruffling (10). Since AA-derived species and AA metabolism generate a state of oxidant stress contributing to liver injury, we measured the extent of prooxidant stress in KC. AA increased intra- and extracellular LPO, H₂O₂, and O₂•− more in KC Ethanol than in KC Control, most likely by enhancing the effect of ethanol. Addition of antioxidants, NADPH inhibitors, and COX and LOX inhibitors prevented these effects. There was an increase in the activity of the antioxidant enzymes by AA in KC, likely suggesting that upregulation of the antioxidant defense was necessary to allow KC to cope with cellular stress. AA minimally altered xanthine oxidase and CYP2E1 activities and did not change ATP levels (46); however, it greatly activated NADPH oxidase, triggering significant ROS generation, which was blocked by a NADPH inhibitor. Thus AA induced oxidant stress via both NADPH oxidase activity and by its own metabolism, effects that were more apparent in KC Ethanol than in KC Control.
To determine the specificity of AA in mediating the abovementioned effects, we incubated cells with EPA, an n-3 PUFA, and with OA, a monounsaturated fatty acid, the latter with antioxidant properties. Both AA and EPA increased oxidative stress, indicating a role for both series of PUFAs compared with a monounsaturated fatty acid, in mediating ROS generation in KC. Moreover, ROS induction by PUFAs was increased by ethanol, thus indicating that the effects of PUFAs and ethanol are additive.

Because of its prominent role in the pathophysiology of ALD (10, 37, 41), we next evaluated TNFα production in KCControl and KCEthanol under AA treatment. TNFα increased significantly and rapidly by AA challenge along with ROS generation, and these effects were more apparent in KCEthanol than in KCControl. Antioxidants, NADPH oxidase inhibitors, and COX and LOX blocking agents prevented the TNFα increase by AA. Thus AA activation of NAPDH oxidase as well as AA metabolism played a role in regulating TNFα in KC.

Since stress-activated kinases are sensitive to prooxidants, we hypothesized that they could play a potential role in the AA induction of TNFα. Several studies (5, 18–19, 36) have identified the MAPK family ERK1/2 as a key target for ethanol in KC. Of all the stress-sensitive kinases analyzed under AA challenge, there was only an increase in pERK1/2, which correlated with the TNFα upregulation. The increase was significantly higher in KCEthanol than in KCControl. Because coinubcation with PD098059 blunted the increase in TNFα by AA, we concluded that pERK1/2 was an upstream kinase regulating TNFα production.

We (10) previously reported that ethanol and AA synergize to activate KC; thus we evaluated the involvement of chronic alcohol feeding in the ROS-driven pERK1/2-mediated upregulation of TNFα. ROS production increased more in KCEthanol than in KCControl (10, 37, 40) as occurred for TNFα. Moreover, ERK1/2 phosphorylation was higher in KCEthanol than in KCControl exposed to AA. These results, consistent with previous work by other laboratories, including ours (10, 36, 38), suggest a role for ROS in activating the pERK1/2 pathway and confirm that ethanol increases the AA-mediated effects on KC-derived TNFα production.

Egr1 is a member of the immediate early gene family and plays a role in mediating cellular responses to environmental stress such as ischemia, mechanical injury, and ionizing radiation (18, 34). Egr1 binding to the Tnfα promoter is required for full activation of TNFα transcription (31–32, 47). Egr1 was rapidly induced after AA challenge more significantly in KCEthanol than in KCControl suggesting that AA drives TNFα induction in an Egr1-dependent manner and that ethanol synergizes with AA to this effect. To conclude, a link between pERK1/2 and Egr1 was established when PD098059 blocked Egr1 induction and TNFα increase. Likewise, a connection between Egr1 and TNFα upregulation was made when curcumin, which prevents nuclear Egr1 translocation (8, 14), blunted the TNFα upregulation by AA.

In summary, the data (Fig. 8) suggest that the AA-driven increase in NAPDH oxidase and AA metabolism per se contribute to elevate ROS generation by KC and that ethanol synergizes to
further enhance these events. Because of the oxidative burst, KC respond inducing ERK1/2 phosphorylation and translocating Egr1 into the nucleus to increase TNFα, therefore, contributing to alcohol-mediated liver damage.

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Fig. 5. AA Upregulates TNFα in KC. A time-course experiment was carried out for intracellular TNFα production by KCControl (dotted line) and KCEthanol (solid line) following AA exposure up to 24 h (A). Under the same culture conditions, secreted TNFα was evaluated by ELISA (B). KCControl and KCEthanol were incubated from 0 to 24 h with 0–10 μM AA (control), 50 μM vitamin E, 200 U/ml of catalase, or both and intracellular TNFα was measured by flow cytometry (C). Cells were also cotreated with 0–10 μM AA (control), 10 μM DPI, or 300 μM apocynin and NADPH oxidase inhibitors, and intracellular TNFα was determined by flow cytometry (D). Likewise, cells were coincubated with 0–10 μM AA (control), 0–10 μM NS398, a COX inhibitor, and 0–10 μM NDGA, a LOX inhibitor, and intracellular TNFα was determined by flow cytometry (E). In A–E, results are calculated as fold change over the nontreated KCControl and are expressed as means ± SE for n = 6. *P < 0.05, **P < 0.01, and ***P < 0.001 for AA treated vs. control; *P < 0.05, **P < 0.01, and ***P < 0.001 for antioxidant or inhibitor treated or cotreated vs. control; *P < 0.05, **P < 0.01, and ***P < 0.001 for KCEthanol vs. KCControl.
Fig. 6. Treatment with AA induces ERK1/2 phosphorylation but no changes in JNK or p38. Western blot analysis of total and pERK1/2 in KCControl (dotted line) and KCEthanol (solid line) treated with 0–10 μM AA up to 24 h. Densitometry is shown at the top and a representative blot for KCEthanol at the bottom (A). KCControl and KCEthanol were incubated with 10 μM DPI or 50 μM vitamin E before AA exposure. Western blot shows a decrease in ERK1/2 phosphorylation by vitamin E and to a lesser extent by DPI in both KCControl and KCEthanol (B). In A and B, results are expressed as arbitrary densitometry units under the blots and are average values of n = 6; the quantification of the signal is referred to total ERK1/2. KCControl and KCEthanol were incubated with 0–1 μM PD098059, a MEK1/2 inhibitor, 1 h before 0–10 μM AA treatment, and intracellular TNFα was determined at 1 and 24 h (C). Cells were also cotreated with AA, and expression of ERK1/2, JNK, and p38 was analyzed by Western blot. GAPDH was used as the loading control (D). Results are calculated as fold change over the nontreated KCControl and are expressed as means ± SE for n = 6. *P < 0.05, **P < 0.01, and ***P < 0.001 for AA-treated vs. control; *P < 0.05, **P < 0.01, and ***P < 0.001 for DPI, vitamin E or PD098059 treated or cotreated vs. control; *P < 0.05, **P < 0.01 and ***P < 0.001 for KCEthanol vs. KCControl.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.J.C. performed experiments; F.J.C. and N.N. analyzed data; F.J.C. and N.N. interpreted results of experiments; F.J.C. and N.N. prepared figures; F.J.C. and N.N. drafted manuscript; F.J.C. and N.N. edited and revised manuscript; N.N. conception and design of research; N.N. approved final version of manuscript.

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