Acetoacetate Activation of Extracellular Signal-Regulated Kinase 1/2 and p38 Mitogen-Activated Protein Kinase in Primary Cultured Rat Hepatocytes: Role of Oxidative Stress

Mohamed A. Abdelmegeed, Sang K. Kim, Kimberley J. Woodcroft, and Raymond F. Novak

Institute of Environmental Health Sciences, Wayne State University, Detroit, Michigan

Received February 5, 2004; accepted March 29, 2004

ABSTRACT

Diabetes is characterized by elevated levels of ketone bodies acetoacetate (AA) and 3-hydroxybutyrate (3HB). High levels of ketone bodies have been implicated in generation of cellular oxidative stress. Ketone body activation of cellular signaling pathways associated with oxidative stress, however, has not been established. Thus, ketone body effects on kinase activation in primary cultured rat hepatocytes have been examined. Treatment with AA increased the phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2) and p38 mitogen-activated protein kinase (MAPK), maximally by 2.5- and 4-fold, respectively. AA failed to activate c-Jun NH2-terminal kinase. AA-mediated Erk1/2 and p38 MAPK activation was detectable at 3 h post-treatment with maximal activation occurring at 12 h. In contrast, 3HB failed to activate any of these kinases. Elevated phosphorylation of Raf and MKK3/6 also occurred in response to AA. Bisindolylmaleimide, a generalized protein kinase C (PKC) inhibitor, and B581, a Ras farnesylation inhibitor, inhibited AA-mediated activation of Erk1/2 and p38 MAPK, suggesting a role for PKC and Ras in mediating such activation. Interestingly, the tyrosine kinase inhibitor genistein prevented the AA-mediated phosphorylation of Erk1/2, but not p38 MAPK. AA treatment resulted in the generation of reactive oxygen species (ROS) and the depletion of cellular glutathione levels, which was ameliorated by the antioxidants N-Acetyl-L-cysteine (NAC) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid). NAC and Trolox also ameliorated AA-mediated Erk1/2 and p38 MAPK activation, suggesting that this activation is associated with ROS and oxidative stress.

Diabetes mellitus is considered the most common cause of elevated levels of ketone bodies (Laffel, 1999). The two major ketone bodies are acetoacetate (AA) and 3-hydroxybutyrate (3HB) (Laffel, 1999). Ketone bodies are produced in the liver and have been postulated to be an emergency gluconeogenesis pathway (Casazza et al., 1984). In humans, blood plasma concentrations of ketone bodies (AA plus 3HB) greater than 1 mM are considered to be hyperketonemic, and these concentrations can reach up to 10 mM in severe ketosis in comparison with concentrations less than 0.5 mM in normal individuals (Jain et al., 2003).

AA, but not 3HB, has been reported to increase lipid peroxidation and growth inhibition in cultured human endothelial cells (Jain et al., 1998). Furthermore, AA has been reported to increase lipid peroxidation and decrease glutathione (GSH) levels in human erythrocytes and type I diabetic (Jain and McVie, 1999).

Several signal transduction pathways, including the mitogen-activated protein kinase (MAPK) superfamily, which consists of extracellular signal-regulated kinase (Erk), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) (Torres, 2003), are known to be activated by oxidative stress. Erk1/2, the best studied in the group of Erks, is activated by MAPK/Erk kinase (Mek). The Meks are activated by the upstream c-Raf, which is the MAPK kinase kinase of this signaling pathway, which in turn is generally activated in response to growth

Received February 5, 2004; accepted March 29, 2004

ABBRVIATIONS: AA, acetoacetate; 3HB, 3-hydroxybutyrate; GSH, glutathione; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; Mek, mitogen-activated protein kinase kinase; PKC, protein kinase C; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; PD98059, 2’-amino-3’-methoxy-flavone; B581, N-(2(S)-(2(R)-amino-3-mercaptopropylamino)-3-methyl(butyl)-Phe-Met-OH; DCFDA, 2’,7’-dichlorodihydrofluorescein diacetate; DCF, 2’7’-dichlorodihydrofluorescein; TBS-T, Tris-buffered saline/Tween 20; GST, glutathione S-transferase; NF-κB, nuclear factor-κB.
factor receptor tyrosine kinase through Ras (Torres, 2003). p38 MAPK and JNK are generally activated by environmental stress and inflammatory cytokines. MKK3/6 is the direct upstream kinase of p38 MAPK, whereas MKK4/7 activates JNK (Torres, 2003). GTPases play an essential role in the transmission of stress stimuli to the MAPK kinase kinases of these signaling pathways (Torres, 2003). There is considerable evidence that PKC can modulate Erk1/2 (Cacace et al., 1996; Perletti et al., 1996) and p38 MAPK activity (Min et al., 2002). The activation of these signaling pathways, however, has not been characterized in response to ketone bodies in primary cultured rat hepatocytes.

The objective of this study was to examine the hypothesis that AA activates MAPK signaling pathways in primary cultured rat hepatocytes and that this activation might be mediated through increased oxidative stress. We demonstrate that AA, but not 3HB, activates Erk1/2 and p38 MAPK signaling pathways in primary cultured rat hepatocytes. We also show that AA increases intracellular reactive oxygen species (ROS) levels and decreases GSH levels. We then provide evidence that the AA-mediated MAPK activation may result from enhanced oxidative stress, because antioxidants N-acetyl-l-cysteine (NAC) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) ameliorated AA-mediated inhibition of GSH levels and prevented the AA-mediated activation of Erk1/2 and p38 MAPK.

Materials and Methods

Chemicals. Modified Chee’s medium and l-glutamine were obtained from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo-Nordisk (Princeton, NJ). Collagenase (type I) was obtained from Worthington Biochemicals (Freehold, NJ). Vitrogen (95–98% type I collagen, 2–5% type III collagen) was purchased from Cohesion Technologies (Santa Clara, CA). Anti-Erk1/2, anti-p38 MAPK, anti-JNK, anti-Raf, anti-MKK3/6, anti-phospho (Thr 202, Tyr 204) Erk1/2, anti-phospho (Thr 180, Tyr 182) p38 MAPK, anti-phospho (Thr 183, Tyr 185) JNK, and anti-phospho (Ser 328) Raf, anti-phospho (Ser 189/207) MKK3/6 were obtained from Cell Signaling Technology Inc. (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Bio-Rad ( Hercules, CA). Enhanced chemiluminescence reagents were obtained from Amersham Biosciences Inc. (Piscataway, NJ). PD98059 and c-Src kinase inhibitors for 6 or 12 h. Hepatocytes were incubated with 20 mM AA, γ-hydroxybutyrate, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Primary Rat Hepatocyte Culture. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200–300 g) using collagenase perfusion as described previously (Woodcroft and Novak, 1997, 1999). Hepatocytes were plated onto dishes coated with collagen in modified Chee’s medium fortified as described previously (Woodcroft and Novak, 1997, 1999) and supplemented with 0.1 μM dexamethasone and 1 μM insulin. Cells were plated at a density of 3 × 10⁶ cells/60-mm dish. To examine the effects of ketone bodies on cellular signaling pathways without interference from insulin activation of examined pathways, 4 h after plating, medium was replaced with insulin-free medium for the duration of the culture. Medium was changed every 24 h. Treatment with ketone bodies (0.05–10 mM), NAC (5 mM), Trolox (1–2 mM), and all kinase inhibitors (5–50 μM) was initiated 48 h after plating, and continued for up to 24 h. NAC, Trolox, and kinase inhibitors were added 1.5 h before treatment with AA. The inhibitors were used at concentrations previously determined by our laboratory to be nontoxic (Woodcroft et al., 2002).

Immunoblot Analysis. To determine the phosphorylation state of Erk1/2, p38 MAPK, JNK, Raf, and MKK3/6, cell lysates were prepared by scraping the cells directly into 500 μl of Laemml sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). Cell lysates (10–15 μl) were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk powder in Tris-HCl buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then probed with phospho-specific antibodies (diluted 1:500 in 5% bovine serum albumin in TBS-T) overnight at 4°C, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in 5% milk powder in TBS-T). Proteins were detected by enhanced chemiluminescence on Kodak X-Omat film (Sigma-Aldrich) and quantified by densitometry with a Molecular Dynamics (Sunnyvale, CA) scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences Inc.). After probing with phospho-specific antibodies, blots were stripped and reprobed for total levels of the respective kinases, and relative levels of phosphorylated kinases were normalized to the total levels of the respective kinase.

GSH Level Determination. To determine total cellular GSH concentrations, cells were washed once in ice-cold phosphate-buffered saline and then scraped into ice-cold 6% perchloric acid. The lysates were transferred into Eppendorf tubes and were clarified by centrifugation at 8000g for 10 min at 4°C. The supernatant was used for determination of total GSH, and the pellets were used for protein determination. For measurement of GSH concentration in medium, 200 μl of medium was transferred into Eppendorf tubes containing 20 μl of 6% perchloric acid. GSH was measured according to an enzymatic recycling method (Griffith, 1980).

Measurement of Intracellular ROS. Intracellular ROS production was monitored by oxidation of DCFDA, which reacts with ROS to form the fluorescent product 2′,7′-dichlorodihydrofluorescein (DCF) (Qu et al., 2001). Hepatocytes (4.5 × 10⁴ cells/well) were cultured in 12-well plates for 48 h before the addition of AA or inhibitors for 6 or 12 h. Hepatocytes were incubated with 20 μM DCFDA for 1 h, washed twice with Hanks’ balanced salt solution, and fluorescence determined (excitation 503 nm, emission 529 nm) using a Molecular Devices Spectramax Gemini spectrofluorometer. ROS levels were reported as DCF fluorescence intensity in response to AA relative to untreated controls.

Statistical Analysis. Significant differences between groups were determined by analysis of variance followed by a multiple comparison test (P < 0.05). Statistical analysis was performed on triplicate cell lysates from a single hepatocyte preparation. Reproducibility of results was confirmed in two to four separate hepatocyte preparations.

Results

To determine whether ketone body treatment of primary cultured rat hepatocytes results in activation of MAPK signaling pathways, Erk1/2 phosphorylation in response to AA or 3HB was examined (Fig. 1). AA (5 mM) failed to increase Erk1/2 phosphorylation at an early time point (10 min), whereas AA treatment for 3 and 12 h resulted in a 1.5- and 2.5-fold increase in Erk1/2 phosphorylation, respectively (Fig. 1A). The phosphorylation of Erk1/2 was maintained for up to 24 h post-treatment relative to control (Fig. 1A). Because Erk1/2 phosphorylation was maximally elevated after 12-h
treatment with AA, this treatment time was used for all subsequent experiments monitoring Erk1/2 activation.

To examine the concentration dependence of AA on Erk1/2 activation, concentrations of AA ranging from normal to ketotic (0.05–5 mM) were used (Fig. 1B). AA at the normal physiological concentration of 0.05 mM did not increase Erk1/2 phosphorylation (Fig. 1B). Erk1/2 phosphorylation increased maximally ~2.5-fold in response to 5 mM AA. Both 0.5 and 1 mM AA increased Erk1/2 phosphorylation by 2-fold relative to control (Fig. 1B). These data show that AA, but not 3HB, activates Erk1/2 and that the activation is maximally induced by the ketotic level of 5 mM AA at 12 h after addition to the primary cultured rat hepatocytes.

We next examined the effect of AA and 3HB on p38 MAPK phosphorylation in primary cultured rat hepatocytes (Fig. 2). AA (5 mM) produced a ~2.5- to 4-fold increase in p38 MAPK phosphorylation at 3 to 12 h, respectively, relative to control (Fig. 2A). The p38 MAPK phosphorylation levels returned to control levels after 24-h treatment (Fig. 2A). These data show that AA, but not 3HB, activates p38 MAPK and that the activation is maximal after 12-h treatment with 5 mM AA. So, AA treatment for 12 h was used in all subsequent experiments to monitor p38 MAPK phosphorylation.

To investigate whether ketone body treatment also activates JNK in primary cultured rat hepatocytes, JNK phosphorylation was examined after treatment with 5 mM AA or 3HB. No significant change in the basal levels of JNK phosphorylation occurred in response to either AA or 3HB (data not shown).

Because AA and 3HB are both elevated during diabetes and may combine to cause synergistic effect, we examined Erk1/2 and p38 MAPK phosphorylation in response to combined treatment with AA (5 mM) and 3HB (5 mM) (Fig. 3). The levels of Erk1/2 phosphorylation in response to AA were similar in the presence or absence of 3HB, relative to control

The concentration dependence of AA on the phosphorylation of p38 MAPK was examined (Fig. 2B). AA concentrations ≤1 mM failed to affect p38 MAPK phosphorylation, whereas 5 mM AA resulted in an ~4-fold increase in p38 MAPK phosphorylation relative to control (Fig. 2B). These data show that AA, but not 3HB, activates p38 MAPK and that the activation is maximal after 12-h treatment with 5 mM AA. So, AA treatment for 12 h was used in all subsequent experiments to monitor p38 MAPK phosphorylation.

To investigate whether ketone body treatment also activates JNK in primary cultured rat hepatocytes, JNK phosphorylation was examined after treatment with 5 mM AA or 3HB. No significant change in the basal levels of JNK phosphorylation occurred in response to either AA or 3HB (data not shown).

Because AA and 3HB are both elevated during diabetes and may combine to cause synergistic effect, we examined Erk1/2 and p38 MAPK phosphorylation in response to combined treatment with AA (5 mM) and 3HB (5 mM) (Fig. 3). The levels of Erk1/2 phosphorylation in response to AA were similar in the presence or absence of 3HB, relative to control
Similarly, the combined treatment with AA and 3HB resulted in no significant change in the levels of p38 MAPK phosphorylation relative to that resulting from AA alone (Fig. 3B). Thus, 3HB, alone or in combination with AA, was without effect on the MAPKs examined.

We next examined the potential upstream mediators that might be involved in AA-mediated Erk1/2 phosphorylation relative to that resulting from AA alone (Fig. 3B). Thus, 3HB, alone or in combination with AA, was without effect on the MAPKs examined.

PKC has been reported to affect Erk1/2 activation by interacting with several proteins in the Ras/Raf/Mek/Erk cascade (Cacace et al., 1996; Perletti et al., 1996; Kohda et al., 2003). Thus, the broad spectrum PKC inhibitor bisindolylmaleimide (5 μM) was used to examine the possible role of PKC in AA-mediated Erk1/2 activation. As shown in Fig. 4A, pretreatment of cells with bisindolylmaleimide completely abolished the AA-mediated increase in Erk1/2 phosphorylation. Bisindolylmaleimide alone also decreased the basal levels of Erk1/2 phosphorylation, although the decrease of the basal Erk1/2 levels did not reach a significant value relative to control (Fig. 4A). These data support the concept that PKC plays a role in mediating Erk1/2 phosphorylation in response to AA.

The involvement of Raf in AA-mediated Erk1/2 activation was examined via the phosphorylation of c-Raf at serine residue 338, which is considered a critical step for Raf acti-
vation (Mason et al., 1999) (Fig. 4B). AA induced a marked ~2.5-fold increase in Raf phosphorylation compared with control cells (Fig. 4B). However, pretreatment of cells with genistein (50 µM) failed to affect the AA-mediated increase in Raf phosphorylation (Fig. 4B), suggesting a lack of involvement of tyrosine kinases upstream of Raf in the AA-mediated activation of Erk1/2. In contrast, bisindolylmaleimide (5 µM) effectively inhibited AA-mediated Raf phosphorylation (Fig. 4B), suggesting a role for PKC in mediating AA activation of Raf. Together, these results suggest that Raf is also involved in AA-mediated Erk activation and that modulation of Erk activity by PKC occurs upstream of Raf. Collectively, these data suggest that AA activation of Erk1/2 begins in the Ras/Raf/Mek cascade and that PKC plays a role in modulating this activation.

To examine the involvement of pathway components upstream of p38 MAPK, we used genistein (50 µM), B581 (50 µM), and bisindolylmaleimide (5 µM) (Fig. 5). Pretreatment of cells with genistein resulted in marginal inhibition of the AA-mediated increase in p38 MAPK phosphorylation levels (~15%) relative to AA treatment alone (Fig. 5A). Genistein alone produced an ~25% increase in the basal level of p38 MAPK phosphorylation (Fig. 5A), which was not significant. It has been postulated that Ras plays a role in the activation of p38 MAPK (McDermott and O’Neill 1999). Thus, we examined the possible role of Ras in AA-mediated p38 MAPK activation. B581 completely inhibited AA-mediated p38 MAPK phosphorylation (Fig. 5A). B581 alone was without effect on the basal levels of p38 MAPK phosphorylation (Fig. 5A). These data suggest a role of Ras activation in AA-mediated p38 MAPK activation.

PKC also has been implicated as a mediator of p38 MAPK activation (Min et al., 2002). Thus, it was of interest to examine the possible role of PKC in the AA-mediated activation of p38 MAPK. As illustrated in Fig. 5A, pretreatment of cells with bisindolylmaleimide effectively inhibited AA-mediated p38 MAPK phosphorylation. Bisindolylmaleimide alone was without effect on the basal levels of p38 MAPK phosphorylation (Fig. 5A). These data implicate PKC in mediating AA activation of p38 MAPK.

The involvement of MKK3/6, the MAPK kinase directly upstream of p38 MAPK, was investigated by Western blotting for p-MKK3/6. An ~3-fold increase in MKK3/6 phosphorylation levels was monitored in cells treated with AA relative to control (Fig. 5B). Because p38 MAPK has been reported to be activated by PKC without the involvement of MKK3/6 (Igarashi et al., 1999), we examined the effect of PKC inhibition on AA-mediated MKK3/6 phosphorylation. Bisindolylmaleimide effectively inhibited the AA-mediated increase in MKK3/6 levels compared with AA alone (Fig. 5B). Bisindolylmaleimide alone was without effect on the basal levels of MKK3/6 phosphorylation (data not shown). Together, these data suggest that MKK3/6 is involved in AA-mediated p38 MAPK phosphorylation and that Ras and PKC also are involved in AA activation of p38 MAPK and are upstream of MKK3/6.

It has been reported that AA causes oxidative stress and lowers GSH levels in human erythrocytes in vitro and in type 1 diabetic patients (Jain and McVie, 1999). In addition, oxidative stress is known to deplete cellular GSH. Thus, intracellular ROS production (Fig. 6) and cellular GSH depletion (Fig. 7) was monitored in response to AA treatment of primary cultured rat hepatocytes. Because GSH is very abundant in the liver compared with other organs (Lenzen et al., 1996), and because of the sensitivity of the methods used, we used higher concentrations of AA (5–10 mM), which are physiologically ketotic levels, to examine AA effects on ROS production and cellular GSH levels. AA (10 mM) treatment of hepatocytes for 6 or 12 h resulted in a 2- or 2.5-fold elevation of ROS levels, respectively, relative to untreated cells at each time point (Fig. 6). AA treatment resulted in a marked decrease in the levels of cellular GSH in a concentration- and time-dependent manner from 3 to 12 h, relative to control (Fig. 7A). Cellular GSH levels were decreased maximally ~22 or 24% by 5 or 10 mM AA, respectively, after 12-h treatment compared with control cells (Fig. 7A). AA was without effect on cellular GSH levels after 1-h treatment, and GSH levels rebounded to control levels after 24-h AA treat-
ment (Fig. 7A). To exclude the possibility that the decrease in cellular GSH levels could be due to enhanced removal or efflux from the hepatocytes, the effect of AA (5 or 10 mM) on medium GSH levels was monitored (Fig. 7B). The levels of GSH in the medium were unchanged up to 12 h (Fig. 7B). The levels of GSH in medium increased dramatically 24 h after AA treatment; however, this increase did not exceed that monitored in the corresponding control cells (Fig. 7B). AA-mediated GSH depletion was ameliorated by pretreatment with the antioxidants NAC or Trolox (Fig. 7C). NAC or Trolox pretreatment maintained GSH levels at 92 or 86%, respectively, of the level monitored in untreated hepatocytes (Fig. 7C). NAC or Trolox alone was without effect on basal GSH levels (data not shown). Ras has been reported to activate NADPH oxidase, which can lead to increased oxidative stress (Irani and Goldschmidt-Clermont, 1998), and PKC activation can stimulate superoxide formation (Lee et al., 2003). To examine the possible role of Ras or PKC activation in AA-mediated GSH depletion, hepatocytes were pretreated with B581, a Ras farnesylation inhibitor, or bisindolylmaleimide, a PKC inhibitor, before AA treatment. B581 pretreated hepatocytes to which AA (10 mM) was added exhibited a 22% decrease in GSH levels, a value comparable with that monitored in cells treated with AA alone (24% decrease in GSH levels). These data suggest that Ras may not play a role in AA-mediated GSH depletion. In contrast, hepatocytes treated with bisindolylmaleimide plus AA (10 mM) exhibited a 16% decrease in GSH levels, a value slightly lower than that monitored in hepatocytes treated with AA alone (24%), although this was not statistically significant. Neither B581 nor bisindolylmaleimide alone had an effect on the basal levels of GSH (data not shown). This result suggests that activation of PKC may play a role in GSH depletion mediated by AA. Together, these data suggest that AA treatment of primary cultured rat hepatocytes results in increased ROS and oxidative stress, although the exact mechanisms underlying this process need further investigation.

To investigate whether oxidative stress plays a role in AA-mediated activation of Erk1/2 or p38 MAPK, hepatocytes were pretreated with NAC or Trolox (Fig. 8A). NAC or Trolox effectively ameliorated AA-mediated Erk1/2 activation compared with AA treatment alone (Fig. 8A). NAC or Trolox alone marginally inhibited the basal levels of Erk1/2 phosphorylation (Fig. 8A). Similarly, pretreatment of cells with NAC or Trolox effectively prevented AA-mediated activation of p38 MAPK (Fig. 8B). NAC, but not Trolox, resulted in a decrease in basal levels of p38 MAPK phosphorylation (Fig. 8B). These data suggest that oxidative stress plays a role in mediating AA activation of Erk1/2 and p38 MAPK.
and humans (Bellward et al., 1988; Barnett et al., 1990; Song et al., 1990), whereas that of glutathione S-transferases (GSTs) has been reported to be decreased (Thomas et al., 1989). Our laboratory, using primary cultured rat hepatocytes, has shown that insulin, in the absence of other hormonal or metabolic factors, effectively decreases CYP2E1 mRNA and protein levels, whereas glucagon antagonizes this decrease (Woodcroft and Novak, 1997, 1999, Woodcroft et al., 2002). In addition, we have demonstrated that the ketone body AA, but not 3HB, decreases CYP2E1 mRNA levels (Woodcroft et al., 2002). We also have examined the effect of insulin and glucagon on the activity and protein levels of GSTs, a major protective mechanism against oxidative stress (Kim et al., 2003). We have demonstrated, using primary cultured rat hepatocytes, that insulin increased α-class GST protein levels and activities, whereas α- and π-class GST protein levels and activities were decreased by glucagon (Kim et al., 2003). We also found that the mRNA, protein, and activity levels of the catalytic subunit of γ-glutamyl cysteine ligase, which plays a pivotal role in the first step of GSH synthesis, increased in response to insulin in primary cultured rat hepatocytes (S. K. Kim, K. J. Woodcroft, and R. F. Novak, unpublished data). Because CYP2E1 metabolism can result in formation of reactive metabolites and CYP2E1 has been implicated in the generation of tissue-damaging hydroxyl radicals in diabetes (Okhuwa et al., 1995; Leclercq et al., 2000), increased CYP2E1 expression in combination with decreased GST expression during diabetes could contribute to elevated oxidative stress levels.

Diabetes mellitus has been reported to be associated with oxidative stress (Evans et al., 2002), which plays a role in diabetic complications. Ketone body elevation is a common finding in diabetes. Recently, there have been several reports about the ability of the ketone body AA, but not 3HB, to generate reactive oxygen species, cause lipid peroxidation, and decrease glutathione levels in erythrocytes, human endothelial cells, and monocytes (Jain et al., 1998; Jain and McVie, 1999; Jain et al., 1999).

As a continuation of our studies using primary cultured rat hepatocytes to examine metabolic and hormonal effects that are disturbed during diabetes, we examined the singular effect of ketone bodies on the activation of MAPK signaling pathways and the role of oxidative stress in this activation. We report that treatment of primary cultured rat hepatocytes with AA, but not 3HB, leads to an activation of Erk1/2 and p38 MAPK signaling pathways. Our results agree with others who reported that although AA and 3HB are structurally similar, AA has different biological actions than 3HB (Jain et al., 2002, 2003; Woodcroft et al., 2002). It has been suggested that the distinctive actions of AA compared with 3HB may be related to the presence of the 2-keto group in AA (Jain and McVie, 1999).

Hepatic insulin resistance and hepatic steatosis have been reported to be a consequence of GSH depletion and increased lipid peroxidation in diabetic patients (Youssef and McCulloch, 2002; Guarino et al., 2003). Furthermore, it has been reported that lipoperoxidative aldehydes accumulate in liver microsomes and mitochondria at a higher rate in spontaneously diabetic BB/WOR rats than in controls (Traverso et al., 1999). Our results show that AA significantly increased intracellular ROS levels and decreased GSH levels compared with untreated cells. GSH is known to scavenge oxidative radicals and maintain reduced thiol homeostasis in cells. Hence, this decrease in GSH levels can further hamper cellular defense against cellular oxidative stress. CYP2E1 has been suggested as playing a role in the metabolism of ketone bodies (Casazza et al., 1984). Whether AA metabolism by CYP2E1 results in increased oxidative stress remains to be established. In addition, we demonstrated that NAC, a thiol-containing antioxidant that can replenish cellular levels of GSH, and the vitamin E analog Trolox, which protects

**Discussion**

Diabetes mellitus is associated with metabolic disturbances, which are found in the metabolism of carbohydrates, lipids, proteins, and also in xenobiotic metabolism. The levels of cytochromes P450 (CYP) 2E1, 2B, 3A, and 4A activity and protein have been reported to increase in diabetic animals and humans (Bellward et al., 1988; Barnett et al., 1990; Song et al., 1990), whereas that of glutathione S-transferases (GSTs) has been reported to be decreased (Thomas et al., 1989). Our laboratory, using primary cultured rat hepatocytes, has shown that insulin, in the absence of other hormonal or metabolic factors, effectively decreases CYP2E1 mRNA and protein levels, whereas glucagon antagonizes this decrease (Woodcroft and Novak, 1997, 1999, Woodcroft et al., 2002). In addition, we have demonstrated that the ketone body AA, but not 3HB, decreases CYP2E1 mRNA levels (Woodcroft et al., 2002). We also have examined the effect of insulin and glucagon on the activity and protein levels of GSTs, a major protective mechanism against oxidative stress (Kim et al., 2003). We have demonstrated, using primary cultured rat hepatocytes, that insulin increased α-class GST protein levels and activities, whereas α- and π-class GST protein levels and activities were decreased by glucagon (Kim et al., 2003). We also found that the mRNA, protein, and activity levels of the catalytic subunit of γ-glutamyl cysteine ligase, which plays a pivotal role in the first step of GSH synthesis, increased in response to insulin in primary cultured rat hepatocytes (S. K. Kim, K. J. Woodcroft, and R. F. Novak, unpublished data). Because CYP2E1 metabolism can result in formation of reactive metabolites and CYP2E1 has been implicated in the generation of tissue-damaging hydroxyl radicals in diabetes (Okhuwa et al., 1995; Leclercq et al., 2000), increased CYP2E1 expression in combination with decreased GST expression during diabetes could contribute to elevated oxidative stress levels.

Diabetes mellitus has been reported to be associated with oxidative stress (Evans et al., 2002), which plays a role in diabetic complications. Ketone body elevation is a common finding in diabetes. Recently, there have been several reports about the ability of the ketone body AA, but not 3HB, to generate reactive oxygen species, cause lipid peroxidation, and decrease glutathione levels in erythrocytes, human endothelial cells, and monocytes (Jain et al., 1998; Jain and McVie, 1999; Jain et al., 1999).

As a continuation of our studies using primary cultured rat hepatocytes to examine metabolic and hormonal effects that are disturbed during diabetes, we examined the singular effect of ketone bodies on the activation of MAPK signaling pathways and the role of oxidative stress in this activation. We report that treatment of primary cultured rat hepatocytes with AA, but not 3HB, leads to an activation of Erk1/2 and p38 MAPK signaling pathways. Our results agree with others who reported that although AA and 3HB are structurally similar, AA has different biological actions than 3HB (Jain et al., 2002, 2003; Woodcroft et al., 2002). It has been suggested that the distinctive actions of AA compared with 3HB may be related to the presence of the 2-keto group in AA (Jain and McVie, 1999).

Hepatic insulin resistance and hepatic steatosis have been reported to be a consequence of GSH depletion and increased lipid peroxidation in diabetic patients (Youssef and McCulloch, 2002; Guarino et al., 2003). Furthermore, it has been reported that lipoperoxidative aldehydes accumulate in liver microsomes and mitochondria at a higher rate in spontaneously diabetic BB/WOR rats than in controls (Traverso et al., 1999). Our results show that AA significantly increased intracellular ROS levels and decreased GSH levels compared with untreated cells. GSH is known to scavenge oxidative radicals and maintain reduced thiol homeostasis in cells. Hence, this decrease in GSH levels can further hamper cellular defense against cellular oxidative stress. CYP2E1 has been suggested as playing a role in the metabolism of ketone bodies (Casazza et al., 1984). Whether AA metabolism by CYP2E1 results in increased oxidative stress remains to be established. In addition, we demonstrated that NAC, a thiol-containing antioxidant that can replenish cellular levels of GSH, and the vitamin E analog Trolox, which protects
against lipid peroxidation, ameliorated AA-mediated inhibition of cellular GSH levels, indicating that oxidative stress and lipid peroxidation play a role in AA-mediated GSH depletion. However, we cannot exclude other mechanisms of AA-mediated GSH depletion. We have shown that NAC and Trolox decreased AA-mediated phosphorylation of Erk1/2 and p38 MAPK. Thus, our results suggest that oxidative stress is responsible, at least in part, for the AA-mediated activation of Erk1/2 and p38 MAPK in primary cultured rat hepatocytes. Whether the activation of these signaling pathways plays a role in hepatic insulin resistance during diabetes requires further investigation.

We have demonstrated that AA activates Erk1/2 and p38 MAPK, but not JNK, in primary cultured rat hepatocytes. Fumarylacetoacetate, a precursor of AA, has been reported to activate Erk, but not p38 MAPK or JNK, in HT1 fibroblasts (Jorquera and Tanguay, 2001). Also, p38 MAPK has been implicated in mediating AA-induced tumor necrosis factor-α secretion in cultured U937 monocytes and in type 1 diabetic patients (Jain et al., 2002). Both studies suggested a possible role of oxidative stress in mediating the activation of these signaling pathways in response to fumarylacetoacetate or AA, which is in agreement with our findings.

Some studies have demonstrated a role of elevated oxidant radicals (Cimino et al., 1997) and decreased GSH levels (Gopalakrishna and Jaken, 2000) in modulating intracellular signaling pathways. MAPK signal transduction pathways are activated by oxidative stress (Torres, 2003). In addition, Ras is a common signaling target of reactive free radicals and cellular redox stress, as evident in the human T cell line Jurkat (Lander et al., 1995). Furthermore, GSH depletion has been reported to regulate Ras/MAPKs in rat mesangial cells (Sandau et al., 1999). Alternatively, Ras has been reported to activate NADPH oxidase, which can lead to increased oxidative stress (Irani and Goldschmidt-Clermont, 1998). We have shown that B581, an inhibitor of Ras farnesylation, was without effect on AA-mediated GSH depletion, suggesting that GSH depletion by AA may not be the result of increased superoxide radicals through Ras activation. On the other hand, we have demonstrated that B581 ameliorated the AA-mediated activation of Erk1/2 and p38 MAPK activation, suggesting a role of Ras in mediating such activation. Our results also support an important role of Mek in mediating the AA effect on Erk1/2 phosphorylation; however, the inhibition was not complete with the Mek inhibitor PD98059. This may be due to partial metabolism of the inhibitor by hepatocytes.

In this study, genistein inhibited the AA-mediated activation of Erk1/2, but not AA-mediated Raf activation. AA may activate Raf by another modulator, such as PKC. PKC has been reported to modulate the phosphorylation of Erks through Raf activation. It has been demonstrated that overexpression of PKC in NIH3T3 resulted in phosphorylation of Raf (Cacace et al., 1996). Furthermore, there was marked increase of Raf-1 phosphorylation in PKC-transformed colon epithelial cells (Perletti et al., 1996). Indeed, our results suggest a role of PKC in AA-mediated Erk1/2 phosphorylation and that PKC is upstream to Raf.

Our results illustrate that PKC also plays a role in AA-mediated p38 MAPK activation. The role of PKC in modulating p38 MAPK is established. For example, PKC activation and subsequent contraction of lower esophageal sphincter circular smooth muscle has been suggested to be due to p38 MAPK activation (Kohda et al., 2003). We further showed that the action of PKC is upstream to MKK3/6.

Oxidative stress has been reported to activate PKC (Finkel, 2003). Also, the activation of PKC can lead to the production of superoxide radicals (Lee et al., 2003). Our results suggest that AA-mediated PKC activation may play a role in AA-mediated GSH depletion. In addition, the role of PKC in modulating Erk1/2 and p38 MAPK is well established. Together, we suggest that the modulation of AA activation of Erk1/2 and p38 MAPK by PKC in the present study is related at least partially with AA-induced oxidative stress.

The activation of MAPK signaling pathways results in the phosphorylation of their downstream target proteins, such as transcription factors, which will ultimately alter cellular function. AA has been implicated in the activation of the nuclear transcription factor nuclear factor-κB (NF-κB) (Hoffman et al., 2002). Our preliminary data indicate that AA treatment of primary cultured rat hepatocytes decreased inhibitory (I) B levels, implicating the activation of NF-κB (our unpublished data).

In conclusion, our results demonstrate for the first time that the activation of Erk1/2 and p38 MAPK signaling pathways is confined to AA and is not a generalized ketone body effect, in primary cultured rat hepatocytes. Furthermore, our data suggest that this activation may be accomplished through events mediated by oxidative stress, which illustrates that ketosis can be considered as a risk factor in the development of oxidative stress in hepatic tissue during diabetes. The identification of the mechanism(s) of AA-mediated oxidative stress and activation of downstream targets, such as transcription factors or genes, will bring new insights in the biochemical mechanism through which cells respond to AA.

References

secretion by acetacetate-treated cultured U937 monocytes. *Diabetes Care* **26:** 2135–2143.


Address correspondence to: Dr. Raymond F. Novak, Institute of Environmental Health Sciences, Wayne State University, 2727 S Ave., Rm 4000, Detroit, MI 48201. E-mail: r.novak@wayne.edu