Differential regulation of the JNK/AP-1 pathway by S-adenosylmethionine and methylthioadenosine in primary rat hepatocytes versus HuH7 hepatoma cells

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Submitted 22 June 2005; accepted in final form 30 January 2006

INTRACELLULAR SIGNALING PATHWAYS are involved in the regulation of cell functions, including programmed cell death or apoptosis, and exert their actions in a pleiotropic fashion. JNK, a member of the superfamily of MAPK characteristicly activated by stress stimuli, like ultraviolet light, TNF-α, or different death receptors, is a mediator of apoptosis in primary hepatocytes and other cell types. Unlike TNF-α or Fas ligand, OA induces apoptosis in the absence of inhibitors of protein synthesis, in a pathway that involves cytochrome c release from mitochondria and caspase 3 activation (1). On the other hand, OA treatment can induce the phosphorylation and activation of JNK through the inhibition of phosphatases involved in the dephosphorylation of the enzyme (17, 40). Some of the effects of OA on gene expression are mediated by the activation of AP-1 (19, 36). However, the requirement of gene expression for the apoptotic effect of OA is still a matter of controversy, because in some cell types OA can induce apoptosis in a protein synthesis-independent way (9), whereas in others the presence of inhibitors of transcription or translation prevents its apoptotic action (15).

We have previously demonstrated that S-adenosylmethionine (AdoMet) and its derivative 5′-methylthioadenosine (MTA) exert a protective action on apoptosis induced by okadaic acid in primary rat hepatocytes but not in human transformed HuH7 cells. In the present work, we analyzed the role played by the JNK/activator protein (AP)-1 pathway in this differential effect. Okadaic acid induced the phosphorylation of JNK and c-Jun and the binding activity of AP-1 in primary hepatocytes, and pretreatment with either AdoMet or MTA prevented those effects. In HuH7 cells, pretreatment with either AdoMet or MTA did not affect JNK and c-Jun activation or AP-1 binding induced by okadaic acid. In both cell types, p38 was activated by okadaic acid, but neither AdoMet nor MTA presented a significant effect on its activity. Therefore, the differential effect of both AdoMet and MTA on the JNK/AP-1 pathway could explain their antia apoptotic effect on primary hepatocytes and the lack of protection they show against okadaic acid-induced apoptosis in hepatoma cells.

Okadaic acid; c-Jun; apoptosis; activator protein-1

MATERIALS AND METHODS

Reagents. OA and the JNK inhibitor SP-600135 were obtained from Calbiochem (San Diego, CA). The JNK inhibitor JNKI1 was from Alexis Biochemicals (Lausen, Switzerland). AdoMet was obtained from Boehringer Ingelheim (Ingelheim, Germany). MTA and cycloheximide were purchased from Sigma Chemical (St. Louis, MO). Cell culture reagents were from Invitrogen (Carlsbad, CA).

Cell culture and treatments. Experiments were performed in conformity with our institution’s guidelines for the use of laboratory animals and were approved by the University of Navarra Committee of Animal Care. Freshly isolated hepatocytes were obtained by liver collagenase perfusion from male Wistar rats as described elsewhere (21). Cell viability was determined by measuring trypan blue exclusion. Only hepatocytes showing values above 90% of viability were used for further experiments. Isolated hepatocytes were resuspended and plated in MEM supplemented with 10% FBS, antibiotics, and nonessential amino acids. HuH7 cells obtained from American Type

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Culture Collection (Rockville, MD) were cultured in DMEM supplemented with 10% FBS, glutamine, and antibiotics. Six hours after cells were plated, the medium was replaced by MEM or DMEM supplemented with 1% FBS, and treatments were carried out 12 h later.

Primary rat hepatocytes and HuH7 cells were treated for the indicated times with either 20 or 125 nM OA, respectively. When indicated, cells were pretreated with the following compounds: anthra-(1,9-cd)-pyrazole-6-(2H)-one (SP-600125; 8 h, 20 or 50 μM), JNKII (8 h, 10 μM), SB-203580 (30 min, 10 μM) and cycloheximide (30 min, 100 μM), AdoMet (30 min, 4 mM), or MTA (30 min, 500 μM). The viability of hepatocytes treated with AdoMet (4 mM) was evaluated by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and found to be similar to that of control hepatocytes (1.12 ± 0.017 vs. 1.0, fold change, arbitrary units).

Western blot analysis. Primary rat hepatocytes or HuH7 cells were treated as described above and extracted in Triton buffer. The protein concentration of the resultant samples was determined by bicinchoninic acid (BCA), and equal amounts of protein were electrophoresed on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose.
membranes (Amersham Biosciences; Piscataway, NJ). Proteins were detected using specific antibodies for caspase 3 (p17 active form), JNK and its phosphorylated form, and phosphorylated p38 purchased from Cell Signaling Technologies (Beverly, MA) or c-Jun and activating transcription factor (ATF)-2, their phosphorylated forms, and p38 purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After samples were incubated with secondary antibodies conjugated to horseradish peroxidase (Promega; Madison, WI), bound antibodies were detected by enhanced chemiluminiscent system from Roche Molecular Biochemicals. For these experiments, primary rat hepatocytes were seeded on 60-mm plates at a density of 3 × 10^6 cells/dish. Cell death ELISA assays were performed according to the manufacturer’s instructions. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor) was calculated as the ratio between the absorbance values of the samples obtained from treated and control cells.

**Statistical analysis.** All the experiments were performed at least in triplicate. Data were analyzed using the Kruskal-Wallis test to determine differences between all independent groups. When significant differences were obtained (P < 0.05), differences between two groups were tested using the Mann-Whitney U-test.

**RESULTS**

**OA activates the JNK/AP-1 pathway in primary rat hepatocytes.** To determine whether the JNK/AP-1 pathway was involved in the apoptotic action of OA, we first analyzed the phosphorylation levels of JNK in primary hepatocytes treated with OA for different time periods. As shown in Fig. 1, OA induced the phosphorylation of JNK in a time-dependent manner. As shown in Fig. 1A, OA-induced JNK phosphorylation was maximal at 2 h of treatment. To identify the members of the AP-1 family present in the AP-1-DNA complexes, supershift assays were also carried out. As shown in Fig. 2C, c-Jun and JunB, and lower levels of JunD and Fos, were detected using specific antibodies for c-Jun, JunB, JunD, and Fos. These results suggest that the JNK/AP-1 pathway is involved in the apoptotic action of OA in primary rat hepatocytes.

**Fig. 3.** Effect of inhibitors on apoptosis of primary hepatocytes caused by OA. A: effects of SP-600125 (SP), JNKI1, SB-203580 (SB), and cycloheximide (CHX) on oligonucleosomal fragment accumulation induced by OA in primary rat hepatocytes. Cells were incubated with or without 20 nM OA for 8 h, and, as indicated, a pretreatment with 20 or 50 μM SP, 10 μM JNKI1, 10 μM SB, or 100 μM CHX was carried out. The accumulation of oligonucleosomal fragments in the cytoplasm was expressed as the enrichment factor (EF), as described in MATERIALS AND METHODS. Each bar represents the mean ± SD of quadruplicate determinations from at least two independent experiments.

A: effect of SP on caspase 3 activation induced by OA. Cells were pretreated with 50 μM SP and incubated with 20 nM OA for 4 h. Western blot analysis was carried out using a specific antibody for the activated form of caspase 3 (p17 subunit). B: effect of SP on AP-1 binding activity induced by OA in primary rat hepatocytes. Cells were pretreated with 50 μM SP and incubated with 20 nM OA for 4 h. EMSA was carried out using a radiolabeled consensus probe for AP-1.

**Fig. 4.** Effect of JNK and AP-1 inhibitors on oligonucleosomal DNA fragmentation in primary rat hepatocytes caused by OA. A: effects of SP-600125 (SP), JNKI1, SB-203580 (SB), and cycloheximide (CHX) on oligonucleosomal fragment accumulation induced by OA in primary rat hepatocytes. Cells were incubated with or without 20 nM OA for 8 h, and, as indicated, a pretreatment with 20 or 50 μM SP, 10 μM JNKI1, 10 μM SB, or 100 μM CHX was carried out. The accumulation of oligonucleosomal fragments in the cytoplasm was expressed as the enrichment factor (EF), as described in MATERIALS AND METHODS. Each bar represents the mean ± SD of quadruplicate determinations from at least two independent experiments.

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present in the complexes obtained from hepatocytes treated for 4 h with OA.

The JNK/AP-1 pathway is involved in the apoptotic action of OA in primary rat hepatocytes. The involvement of the JNK/AP-1 pathway on the apoptotic effect of OA in primary rat hepatocytes was established using the JNK inhibitor SP-600125. This compound does not alter the phosphorylation of JNK but inhibits its kinase activity in a reversible and competitive way (6). Pretreatment of primary rat hepatocytes with SP-600125 prevented, in a dose-response fashion, the apoptotic effect of OA determined as the accumulation of oligonucleosomal fragments in the cytoplasm of cells. Another pharmacological JNK inhibitor, JNKII, presented a similar effect. Pretreatment with the protein synthesis inhibitor cycloheximide also caused a significant decrease of OA-induced apoptosis, showing that newly synthesized proteins contribute to the apoptotic response elicited by OA. On the contrary, the inhibitor for p38 (SB-203580), a kinase that (like JNK) is activated by stress signals, did not prevent apoptosis induced by OA (Fig. 3A). The effect of SP-600125 was also analyzed on another parameter of apoptosis, caspase 3 activation, which has been shown to be induced by OA in primary rat hepatocytes (1). As expected, JNK inhibition diminished caspase 3 activation in hepatocytes treated with OA (Fig. 3B).

AP-1 binding activity was also evaluated in primary hepatocytes that had been pretreated with SP-600125 before cells were incubated with OA. The JNK inhibitor effectively prevented the binding of AP-1 to its consensus sequence induced by OA (Fig. 3C).

AdoMet and MTA prevent the apoptotic effect of OA through the inhibition of the JNK/AP-1 pathway. Once the role played by the JNK/AP-1 pathway on the apoptotic effect of OA was established, we analyzed its regulation by AdoMet and MTA, two compounds that have been previously shown to abolish apoptosis of hepatocytes induced by OA (1). Pretreatment of primary rat hepatocytes with either AdoMet (4 mM) or MTA (500 μM) prevented the phosphorylation of JNK caused by incubation with 20 nM OA for 4 h without affecting the total content of the protein. The protein c-Jun, a JNK substrate that is phosphorylated in response to OA, presented lower levels of the phosphorylated form in cells pretreated with either AdoMet or MTA, whereas the total protein content was not altered by any of the treatments (Fig. 4A). A similar effect was observed using lower doses of AdoMet (1 mM; data not shown).

The phosphorylation levels of p38 and its substrate ATF-2 were also analyzed in hepatocytes treated for 4 h with OA and pretreated with either AdoMet or MTA. As it was observed for JNK and c-Jun, incubation with OA enhanced the phosphory-

**Fig. 4.** Effect of pretreatment with S-adenosylmethionine (AdoMet) and 5′-methylthioadenosine (MTA) in primary rat hepatocytes incubated with OA. Primary rat hepatocytes were pretreated with AdoMet (4 mM) or MTA (500 μM) and treated with 20 nM OA for 4 h. A: Western blot analysis of JNK, c-Jun, p38, and activating transcription factor (ATF)-2 phosphorylation. Total protein levels were identified using specific antibodies, and their phosphorylation levels were analyzed using antibodies for the phosphorylated forms of the proteins. B: AP-1 binding activity analyzed by EMSA using a radiolabeled consensus AP-1 probe.
lated forms of p38 and ATF-2. However, as shown in Fig. 4A, the phosphorylated forms of p38 and ATF-2 in hepatocytes pretreated with AdoMet or MTA and treated with OA were only slightly lower than those obtained from OA-treated cells.

The effect of AdoMet and MTA in the binding activity of transcription factor AP-1 induced by OA was determined as well. AP-1 binding activity was almost totally abolished in extracts obtained from hepatocytes pretreated with AdoMet and MTA and treated with OA in the same conditions as above compared with OA-treated samples (Fig. 4B).

AdoMet and MTA have no effect on the JNK/AP-1 pathway activated by OA in hepatoma cells. To determine whether AdoMet and MTA had different regulatory actions on the JNK/AP-1 pathway activated by OA in tumor cells, we performed similar experiments to those already described with the human hepatoma cell line HuH7. As observed for primary hepatocytes, treatment of HuH7 cells with OA caused a sustained increase of the phosphorylation levels of JNK (Fig. 5A). Pretreatment with the JNK inhibitor SP-600125 prevented both caspase 3 activation as well as c-Jun phosphorylation induced by OA in HuH7 cells (Fig. 5B).

AP-1 binding activity was also induced in a sustained fashion by treatment with OA, although hepatoma cells presented higher basal levels than hepatocytes (Fig. 6A). Supershift experiments were carried out to determine the composition of both basal AP-1, present in untreated cells, and of AP-1 dimers induced by treatment with OA. AP-1 of control samples was mainly composed of c-Jun and JunD, with lower levels of members of the Fos family (Fig. 6B). Extracts from OA-treated cells had a different pattern of AP-1 members, with higher levels of c-Jun, JunB, and Fos and lower levels of JunD (Fig. 6C).

The effect of AdoMet or MTA on the JNK/AP-1 pathway induced by OA on hepatoma cells was then evaluated. As shown in Fig. 7A, pretreatment of HuH7 hepatoma cells with AdoMet or MTA did not significantly affect the phosphorylation levels of JNK and c-Jun, which were similar to those observed for OA-treated cells, although some differences between both metabolites were detected, with AdoMet having a slight inhibitory effect that was not observed for MTA. The phosphorylation of p38 and ATF-2 induced by OA remained unchanged by pretreatment with either AdoMet or MTA. In the absence of OA, neither AdoMet nor MTA had any effect on the total or phosphorylated protein levels of JNK, c-Jun, p38, or ATF-2 (Fig. 7A).

Similar results were obtained in EMSA analysis of AP-1 binding activity in HuH7 cells treated as above: the induction of AP-1 caused by OA was not altered by pretreatment with AdoMet or MTA, and neither compound had an effect in AP-1 binding activity in the absence of OA (Fig. 7B).

**DISCUSSION**

AdoMet is a metabolite of the essential amino acid methionine involved in cellular transmethylation reactions and a precursor of polyamine and glutathione synthesis in the liver. AdoMet exerts many hepatoprotective effects that are due to different molecular mechanisms, some of them not related to methylation reactions. As a GSH precursor, AdoMet protects liver cells from oxidative stress caused by ethanol metabolism, xenobiotics, or viral infections (25, 30). In apoptosis due to the accumulation of toxic bile acids, the protective role of AdoMet is also related to its antioxidant action (7). However, as we have already shown, the protective effect of AdoMet in apoptosis induced by OA in hepatocytes is not caused by its role as GSH precursor and could be mediated by MTA (1). MTA, which can be formed from decarboxylated AdoMet during polyamine biosynthesis or by the spontaneous splitting of AdoMet under physiological conditions (4), also presents beneficial effects in the liver, including the preservation of hepatocyte viability and prevention of hepatocarcinoma development (28, 34). Interestingly, the synthesis and metabolism of both compounds, AdoMet and MTA, are impaired in chronic liver damage and hepatocarcinoma, and these defects have been associated with the progression of the disease (3, 8, 25, 28, 34). In the present report, we demonstrated that the antiapoptotic action of AdoMet and MTA in primary hepatocytes is due, at least in part, to their capacity in inhibiting the JNK/AP-1 cascade. The stress-activated kinase p38, also activated by OA, was not significantly regulated by AdoMet and MTA, suggesting that JNK is a specific target for the inhibitory action of both compounds. The JNK/AP-1 pathway is involved in many pathophysiological responses of the liver, including alcoholic liver disease and ischemia-reperfusion injury in liver transplant. In this respect, recent studies have shown in both culture systems and animal models of liver transplant that JNK...
inhibition prevents apoptosis of hepatocytes and improves survival (24, 37, 38). JNK activation in the liver is also responsible for insulin resistance and glucose tolerance characteristic of diabetes, and its inhibition exerts beneficial effects in genetic and dietary models of this pathology (27). These data suggest that AdoMet and MTA could be used as JNK inhibitors in pathologies in which JNK activation contributes to cell damage or to the progression of the disease.

Interestingly, AdoMet and MTA have opposite effects depending on the phenotype of the cells: both metabolites present a protective action on apoptosis induced by OA in primary hepatocytes but fail to prevent apoptosis induced by OA in hepatoma cells. Moreover, they also provoke a slight apoptotic effect in the absence of any other apoptotic inducer (1). Therefore, AdoMet and MTA could favor the apoptotic process of malignant cells in two ways: inducing apoptosis of transformed cells by themselves and not preventing the apoptotic action of compounds such as OA, as they do in nontransformed cells. Our results indicate that both effects are independent: apoptosis induced by AdoMet or MTA in hepatoma cells is not due to the activation of the JNK/AP-1 pathway, because neither of them altered the phosphorylation levels of JNK or the binding activity of AP-1. Other mechanisms, like the enhanced expression of the proapoptotic protein Bcl-xS that has been described in HepG2 hepatoma cells, could explain the apoptotic effect of both metabolites in the absence of apoptotic inducers (41).

Our data infer that AdoMet and MTA did not protect HuH7 cells from apoptosis caused by OA because they failed to prevent JNK and AP-1 activation. Previous reports have already described that JNK and c-Jun activation can lead to apoptosis of hepatoma cells (16, 18). Moreover, it has been...
shown that antitumoral agents exert their effects through JNK activation and apoptosis of hepatoma cells (10, 13). Although hepatoma cells present high levels of AP-1, probably related to their proliferative state, increased AP-1 activity and changes in the specific composition of its components induced by JNK activation would act as negative regulators for cell survival. We found that treatment with OA greatly induced the extent of AP-1 binding activity and also altered the composition of AP-1 components. OA-activated AP-1 had higher levels of JunB and lower levels of JunD than AP-1 from control cells. A decrease of JunD could be involved in apoptosis, because this protein has been described to exert antiapoptotic effects (5, 39). On the other hand, an increase in JunB levels could also facilitate apoptosis through inhibition of proliferation by blocking the cell cycle (5, 29). The lack of effect of AdoMet and MTA on the JNK/AP-1 pathway of hepatoma cells would ensure that these metabolites would not interfere in the activation of JNK leading to apoptosis of tumoral cells.

Further studies are needed to establish the molecular mechanism by which AdoMet, and more specifically MTA, which is responsible for the effects of AdoMet in OA-induced apoptosis, prevent the activation of JNK in primary hepatocytes. MTA has been already described to inhibit other kinase activities like tyrosine kinase induced by the fibroblast growth factor receptor and nerve growth factor (23, 26, 35) or p38 activity induced by LPS (14), but the mechanisms of these effects remain to be elucidated. Nevertheless, the possibility of regulating the JNK/AP-1 pathway in primary but not transformed hepatic cells offers new perspectives for the application of AdoMet and MTA in those pathological processes in which JNK is involved.

ACKNOWLEDGMENTS

The authors thank José María Mato for critical reading of this manuscript and Adela Bezumartea and Eva Petri for technical assistance.

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GRANTS

This work was supported by Ministerio de Sanidad y Consumo Fondo de Investigaciones Sanitarias Grants PI030391 (to M. J. Iraburu), PI0408019 (to Barreales), PI020369 (to E. R. García-Trevijano), and PI0510414 and CP04/00123 (to M. A. Ávila). M. A. Ávila was supported by Ministerio de Educación y Ciencia Grant SAF2004-03538, Instituto de Salud Carlos III Grants C03/02 and G03/015, and National Institutes of Health (NIH) Grant ROI AA-12677. E. R. García-Trevijano was supported by NIH Grant R01-AT-1576. This work was also supported by the agreement between Fundación...
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para la Investigación Médica Aplicada and the “Unión Temporal de Empresas project CIMA” and by “becas Ortiz de Landázuri,” Gobierno de Navarra.