Stimulation of DNA synthesis, activation of mitogen-activated protein kinase ERK2 and nuclear accumulation of c-fos in human aortic smooth muscle cells by ketamine

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Abstract. Proliferation of vascular smooth muscle cells is known to be regulated by autocrine and paracrine stimuli, including extracellular matrix, reactive oxygen species, lipids, and biomechanical forces. The effect of many pharmacological agents to which smooth muscle cells may be exposed, however, is widely unexplored. Ketamine, an intravenous anaesthetic and a phencyclidine derivative, regulates diverse intracellular signalling pathways in smooth muscle cells, several of which are known to affect cell proliferation. The effect of ketamine on proliferative response of smooth muscle cells, however, is not determined. We tested the hypothesis that ketamine may regulate proliferation of smooth muscle cells, and investigated the effects of pharmacological doses of ketamine on their proliferative capacity by measuring DNA synthesis and activation of mitogen-activated protein (MAP) kinase signalling pathway in human aortic smooth muscle cells. DNA synthesis, as determined by incorporation of ^3H -thymidine into DNA, was enhanced by 73% ($P < 0.0001$) and 130% ($P < 0.0001$) with 10 and 100 μM ketamine, respectively. Ketamine-induced DNA synthesis was dependent on *de novo* protein synthesis, as it was abolished by an inhibitor of protein synthesis, cycloheximide. A synthetic inhibitor of MAP kinase pathway, PD98059, decreased 50% ($P < 0.0001$) of ketamine-induced DNA synthesis, suggesting that the activation of MAP kinase pathway was partially responsible for ketamine-induced effects. Consistently, in-gel kinase assay and *in vitro* kinase assay of cell lysates showed ketamine-induced MAP kinase activation and expression of ERK2 (extracellular signal-regulated kinase) in smooth muscle cells. This effect of ketamine was not dependent on *de novo* protein synthesis. Immunofluorescent light microscopy showed ketamine-induced nuclear accumulation of c-fos, a downstream effect of MAP kinase activation, in smooth muscle cells. In conclusion, these data support the hypothesis of the study and demonstrate that ketamine, by stimulating DNA synthesis in human aortic smooth muscle cells, may have an impact on proliferative capacity of these cells. The present results also demonstrate that ketamine induces the activation of MAP kinase pathway and nuclear accumulation of transcription factor c-fos in smooth muscle cells. They further demonstrate that the activation of MAP kinases is partially responsible for

ketamine-induced DNA synthesis in human aortic smooth muscle cells. Together, these findings suggest that ketamine may play a role as a pharmacological regulator of mechanisms involved in proliferation of smooth muscle cells.

INTRODUCTION

The proliferation of smooth muscle cells is a key underlying event involved in vascular diseases including atherosclerosis, restenosis following angioplasty, and neonatal pulmonary hypertension (Schwartz *et al.* 1990). The thickening of arterial media and its remodelling during development and progression of atherosclerotic lesions and after a variety of stressful conditions including severe pulmonary hypertension, restenosis following angioplasty, and hypoxic injury, occur as the result of changes in proliferative response of smooth muscle cells (Wohrley *et al.* 1995). Furthermore, proliferation of smooth muscle cells during development and physiological growth of vasculature plays an important role in the maintenance and remodelling of the vascular system (Schwartz *et al.* 1986). The proliferative response of vascular smooth muscle cells may be regulated by autocrine and paracrine stimuli. The paracrine growth mechanisms which have been extensively studied include extracellular matrix, reactive oxygen species, lipids, and biomechanical forces (Berk 2001). The effects of non-protein pharmacological agents, to which smooth muscle cells may be exposed for either short or prolonged durations, however, are widely unexplored.

Ketamine is an intravenous anaesthetic and a phencyclidine derivative, the effect of which, on vascular smooth muscle cells, is expected from its metabolic actions. The effect of ketamine on diverse intracellular pathways involved in cell cycle regulation is established in a variety of cell types. In smooth muscle cells, ketamine induces a decrease in endothelin-1 expression (Hayashi *et al.* 1999). In monolayers of A7r5 vascular smooth muscle cells, ketamine inhibits agonist-induced Ca^{++} release from intracellular stores (Kanmura *et al.* 1996). Furthermore ketamine is shown to inhibit the agonist-induced accumulation of cyclic AMP in human smooth muscle cells (Hill *et al.* 1999). An increased level of cAMP, which causes cell cycle arrest in the G1 and G2/M1 phases, is a known cause of inhibition of smooth muscle cell proliferation (Bornfeldt & Krebs 1999; Hayashi *et al.* 2000). While these effects point to the involvement of ketamine in regulation of intracellular signalling pathways that control cell proliferation, to date, it is unknown whether ketamine has any effects on proliferative response of smooth muscle cells. Therefore, this study was undertaken to test the hypothesis that ketamine may regulate proliferation of smooth muscle cells.

The signal transduction pathways involved in the proliferation of smooth muscle cells are extensively studied but not fully understood. Among the intracellular signalling pathways known to mediate cell cycle progression, the MAP kinase pathway in the smooth muscle cells is the subject of intense investigation. MAP kinases ERK1 and ERK2 (extracellular signal-regulated kinases) are a family of serine/threonine kinases that are activated in response to diverse stimuli involved in cell proliferation and differentiation (Tamemoto *et al.* 1992; Pages *et al.* 1993). The stimulation of expression and activation of MAP kinases in smooth muscle cells by autocrine and paracrine factors including angiotensin II (Duff *et al.* 1992), vasopressin (Granot *et al.* 1993) and endothelin-1 (Koide *et al.* 1992), all with regulatory effects on smooth muscle cell proliferation, have been shown previously. In this study, we also investigated the effect of ketamine on the activation of MAP kinase pathway in smooth muscle cells.

MATERIALS AND METHODS

Cell culture

Human smooth muscle cells (T/G HA-VSMC) were purchased from American Type Culture Collection (ATCC, Rockville, MD). T/G HA-VSMC is a smooth muscle cell line initiated from the normal aorta of an 11-month old Caucasian female. Cells were plated in 24-well (for DNA synthesis) and 60 mm (for kinase, western and immunofluorescent experiments) culture dishes and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 µg/ml insulin, 10 µg/ml transferrin and 10 ng/ml sodium selenite at 37 °C in a humidified incubator with 10% CO₂.

Treatment of cells with chemicals

Cells were grown until confluent and then serum-deprived for 18–24 h before treatment with 10–100 µM ketamine, concentrations which encompass the pharmacological plasma level (Idvall *et al.* 1979). Other chemicals used in this study were: cycloheximide (10 µg/ml), phorbol 12,13-dibutyrate (PDBU) (1 µM) and a synthetic, noncompetitive inhibitor of MAP kinase pathway, PD98059 {[2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]} (Dudley *et al.* 1995; Pang *et al.* 1995) (Calbiochem, La Jolla, CA, USA) 10–20 µM. At this concentration range, PD98059 inhibits the MAP kinase activity in aortic smooth muscle cells by more than 75% (Bornfeldt *et al.* 1997).

DNA synthesis

DNA synthesis was determined by measuring the incorporation of ³H-thymidine (5 µCi/ml), specific activity 86 Ci/mmol (Amersham Life Sciences, Amersham, Buckinghamshire, UK) into DNA of smooth muscle cells in quiescent cultures, as previously described (Sigel *et al.* 1996). Ketamine alone, or in conjunction with cycloheximide or PD98059, was added to the serum-free culture medium 5 min prior to the addition of ³H-thymidine. Cells were then incubated for 1–2 h. At the end of treatment period, cells were washed three times with PBS and lysed in lysis buffer (25% sodium dodecyl sulphate in 10 N NaOH). DNA was precipitated by 5% trichloroacetic acid (TCA). The precipitate was filtered and filters were washed with 5% TCA and 95% ethanol. The radioactivity of the filters was determined by liquid scintillation counting. DNA concentration in the cell lysate was measured by the use of a chromogenic reagent (0.1 M acetaldehyde, 1.5% diphenylamine in glacial acetic acid, and concentrated sulphuric acid) and spectrophotometry as previously described (Sigel *et al.* 1996).

In-gel kinase assay

In-gel kinase assay was performed according to the previously described methods (Sauma & Friedman 1996) and our previous work (Lee & Eghbali-Webb 1998). Confluent, quiescent-smooth muscle cells were incubated with 10 µM ketamine or 1 µM phorbol 12,13-dibutyrate (PDBU) for 5–60 min. The culture medium was aspirated, cells were rinsed three times with ice-cold PBS and lysed (scraped and passed three times through 26 gauge needle) in 0.5 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 2 mM benzamide, and 1% Triton X-100). Cell debris was removed by centrifugation (30 min at 16 000 × g). The supernatant was transferred to a new tube. The concentration of protein in cell lysate was measured using a BCATM protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's guidelines. Aliquots (20 µg protein) of cell lysate were mixed with one half sample volume of 2 × Laemmli buffer (62.5 mM Tris HCl, pH 6.5, 2% SDS, 20% glycerol, 0.01%

bromophenol blue, 5% β -mercaptoethanol), boiled for 5 min, and then electrophoresed in a 10% SDS-polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP). After fixing the gel with four changes of 20% 2-propanol in 50 mM Tris-HCl, pH 7.8 for 2 h, SDS was removed by rinsing the gel in several volumes of 50 mM Tris-HCl, pH 8.0 containing 5 mM 2-mercaptoethanol (2 h, with frequent changes). The kinases were then re-denatured with 6 M guanidine HCl for 2 h and then renatured by incubating the gel overnight with 50 mM Tris-HCl, pH 8.0 containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol. After preincubation for 1 h with 50 ml of 40 mM Hepes, pH 8.0 containing 2 mM 2-mercaptoethanol and 10 mM MgCl_2 , phosphorylation of MBP within the gel was achieved by incubating the gel in 30 ml of 40 mM Hepes, pH 8.0 containing 25 μCi [$\gamma^{32}\text{P}$]ATP, 40 μM ATP, 0.5 mM EGTA, and 10 mM MgCl_2 . The gel was then washed in 5% (w/v) trichloroacetic acid containing 1% sodium pyrophosphate until the radioactivity in the wash reached background levels. The gel was dried in a gel drier at 80 °C and exposed to HyperfilmTM-MP (Amersham). Bands corresponding to phosphorylation signals were analysed by scanning densitometry.

Immunoprecipitation of ERK1 and ERK2 and *in vitro* kinase assay

ERK1 and ERK2 were immunoprecipitated by incubating aliquots of cell lysate corresponding to 200 μg protein with 1 μg of affinity-purified rabbit polyclonal anti-ERK1 or anti-ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 30 μl of Protein A/G-agarose (Santa Cruz) at 4 °C for 3 h. ERK1 or ERK2 activity was assayed as described (Minden *et al.* 1994). Immunocomplexes were washed 5 times with kinase buffer (30 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 1 mM MnCl_2 , and 0.5 mM DTT) and resuspended in 30 μl of kinase cocktail (kinase buffer containing 14 μg MBP, 10 μM ATP, and 5 μCi [$\gamma^{32}\text{P}$]ATP per sample). After 30 min at 30 °C, samples were centrifuged, the supernatant was transferred to a new tube and denatured by boiling 5 min in 1 $\times$ Laemmli buffer and then subjected to electrophoresis on a 15% SDS-polyacrylamide gel. The gel was stained, destained, dried, and exposed to X-ray film at -80 °C. Stained MBP bands were cut and radioactivity was determined by liquid scintillation counting.

Western analysis of tyrosine phosphorylated ERK1 and ERK2

The immunocomplexes (the pellets from above experiment) were washed 5 times with ice-cold lysis buffer and resuspended in 30 μl of 2 $\times$ Laemmli buffer, boiled for 5 min and centrifuged. The supernatant was electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to an ImmobilonTM-P (Millipore Corporation, Bedford, MA, USA) membrane. The membrane was incubated overnight with TBS (Tris-buffered saline) containing 3% dry milk. TBS contained (per l) 8 g NaCl, 0.2 g KCl, and 3 g Tris base and adjusted to pH 7.4 with HCl. Membrane was rinsed with TTBS (TBS containing 0.05% (v/v) Tween 20) twice, incubated for 1 h in TTBS containing 0.2% dry milk and 0.5 $\mu\text{g}/\text{ml}$ of monoclonal antiphosphotyrosine antibody 4G10 (Upstate Biotech, Lake Placid, NY, USA), rinsed with TTBS (15 min with 3 changes at 5 min intervals) and incubated for 1 h with TTBS containing 0.2% dry milk and 1 : 20 000 dilution of a horse radish peroxidase-conjugated goat antimouse IgG (Amersham Life Science, Amersham, UK). After rinsing with TTBS, bands corresponding to tyrosine phosphorylated ERK1 and ERK2 were visualized by enhanced chemiluminescence using an ECLTM (Amersham) kit according to the manufacturer's guidelines.

Immunofluorescent light microscopy

Cells were cultured in DMEM with 10% FBS in 60 mm culture dishes, until confluent. Cells were then serum-deprived for 24 h and treated with ketamine for 1–2 h. The culture medium was then removed, cell layers were washed twice with PBS and permeabilized and fixed by incubation

with 100% methanol for 7 min at 4 °C. Cell layers were air-dried, rehydrated with PBS and incubated with rabbit polyclonal antibodies raised against purified fusion protein corresponding to an N-terminal fragment (amino acid residues 75–155) of murine v-fos-encoded protein (Upstate Biotechnology, Lake Placid, NY, USA) and a synthetic peptide corresponding to a portion of human c-jun protein (Upstate Biotechnology, Lake Placid, NY) at room temperature for 1 h. Cell layers were then washed 4 times with PBS. As negative controls, parallel plates were incubated with IgG prepared from rabbit non-immune serum. Fluorescein isothiocyanate-conjugated goat antirabbit IgG was used as second antibody. Visualization was performed by use of an Olympus, Model BH2 epi-fluorescent microscope (Tokyo, Japan). Photographs were taken using Kodak Tri-X pan 400 film (Rochester, NY, USA).

Statistical analysis

The standard *t*-statistic was used for comparing the results of DNA synthesis in control and treatment groups. Analysis of variance with Bonferroni bounds was used for multiple comparisons. Significance was assumed at $P < 0.05$.

RESULTS

DNA synthesis

DNA synthesis in human aortic smooth muscle cells was determined in the presence and absence of ketamine. The results showed a significant (73%; $P < 0.0001$) stimulation of DNA synthesis with 10 μM ketamine. This effect was enhanced (130%; $P < 0.0001$) with 100 μM ketamine (Fig. 1).

The activation of MAP kinases is a requirement for triggering the proliferative response in most cell types. To determine whether the observed stimulatory effect of ketamine on DNA synthesis involves the MAP kinase pathway, the effect of ketamine on DNA synthesis was determined in the presence of an established inhibitor of MAP kinase cascade, PD98059. The results showed that PD98059 partially, but significantly, inhibited the ketamine-induced increase in DNA synthesis 48% ($P < 0.0001$) and 50% ($P < 0.0001$) inhibition for the effect of 10 and 100 μM ketamine, respectively (Fig. 1).

The involvement of the intracellular signalling mechanisms requiring *de novo* protein synthesis, including those leading to the induction of nuclear transcription factors in ketamine-induced stimulation of DNA synthesis, was tested by measuring the effect of ketamine in the presence of the inhibitor of protein synthesis, cycloheximide. The results showed complete abolishment of ketamine-induced stimulation of DNA synthesis in the presence of cycloheximide (Fig. 1).

Ketamine-induced MAP-kinase activation

The findings, which demonstrated that ketamine-induced DNA synthesis is partially dependent on the activation of MAP kinases, pointed to the possibility that ketamine may directly or indirectly activate MAP kinases in human aortic smooth muscle cells. This possibility was confirmed by the results of in-gel kinase assay which showed increased kinase activity as evidenced by phosphorylation of MAP kinase substrate, MBP, at gel positions corresponding to 42 kDa in ketamine-treated cells over control cells (Fig. 2). Similar to the time course of mitogen- and growth factor-induced MAP kinase activation, the ketamine-induced kinase activity was rapid and transient because it was detectable after 10 min treatment with ketamine and returned to the control levels after 1 h.

The identity of the protein corresponding to the observed kinase activity, was determined by immunoprecipitation of the extracellular signal-regulated kinases, ERK1 (44 kDa) and ERK2

Figure 1. Effect of ketamine on DNA synthesis in smooth muscle cells. Confluent, quiescent human smooth muscle cells were treated with ^3H -thymidine ($5 \mu\text{Ci/ml}$) in the presence and absence of 10 and $100 \mu\text{M}$ ketamine for 1.5 h (a). DNA synthesis was determined by measurement of ^3H -thymidine incorporation as described in Materials and Methods. The effect of MAP kinase inhibitor, PD98059 on ketamine-induced DNA synthesis is shown in (b) and (c). The effect of cycloheximide on ketamine-stimulated DNA synthesis is shown in (d). I-statistics, $n = 4-6$. Bars represent standard error.

(42 kDa) from ketamine-treated smooth muscle cell lysates and subsequent measurement of kinase activity of the immunocomplexes by *in vitro* kinase assay. The results showed a 7-fold increase in ERK2 activation in smooth muscle cell lysate treated with ketamine (10 min) compared to that in the untreated control cells (Fig. 3). ERK1 activity, however, remained unchanged compared with that in the untreated control cells (data not shown).

Since *de novo* protein synthesis was shown to be required for ketamine-induced DNA synthesis, we asked if this protein synthesis is acting upstream of ketamine-induced activation of ERK2 and determined the effect of ketamine on ERK2 activation in the presence of cycloheximide. The results showed that the presence of cycloheximide does not have a significant effect on the activation of ERK2 by ketamine, indicating that *de novo* protein synthesis, which is necessary for ketamine-induced DNA synthesis, is active downstream of ERK2 (Fig. 3).

Figure 2. In-gel MAP kinase assay. Ketamine (10 μ M)-induced MAP kinase activation in confluent quiescent human aortic smooth muscle cells was determined by in-gel kinase assay as described in Materials and Methods. In (a), phosphorylation of MBP at a gel position corresponding to 42 kDa is shown. (b) shows the result of densitometry scanning of the corresponding bands on the autoradiograph. The MAP kinase activation in PDBU (1 μ M)-treated cells is shown as a positive control. These autoradiographs are representatives of three independent experiments.

The activation of ERK2 is directly related to its phosphorylation on Tyr/Thr residues. The ketamine-induced activation of ERK2 was further confirmed by determining the effect of ketamine on ERK2 phosphorylation by western analysis for phosphotyrosyl ERK2. The results showed that 10 min treatment with ketamine increased tyrosine phosphorylation of ERK2 (Fig. 4). Consistent with the results of in-gel kinase and *in vitro* kinase assays, this effect was also transient, because it was not observed in cells treated with ketamine for longer periods (Fig. 4).

Ketamine-induced expression of c-fos

ERK2 activation and its subsequent translocation into the nucleus is known to facilitate the enhanced transcription and activation of c-fos. Our findings with cycloheximide which indicated the involvement of ketamine-induced de novo protein synthesis downstream of ERK2 activation, pointed to the possibility that ketamine-induced signalling may extend to nuclear events in smooth muscle cells. The results of immunofluorescent light microscopy with antifos antibodies confirmed this possibility and demonstrated ketamine-induced nuclear accumulation of c-fos as evidenced by c-fos nuclear staining in ketamine-treated cells (Fig. 5). Nuclear staining was absent in untreated cells (Fig. 5).

Ketamine-treatment did not induce c-jun accumulation in smooth muscle cells (data not shown).

DISCUSSION

The conclusion of the present study is 2-fold: first, ketamine may regulate proliferative response in human vascular smooth muscle cells; second, this effect of ketamine involves intracellular

Figure 3. Immunoprecipitation of ERK2 and subsequent *in vitro* kinase assay of immunocomplexes. Aliquots (200 µg protein) of cell lysate, obtained from smooth muscle cells treated with 10 µM ketamine or 1 µM phorbol 12,13-dibutyrate (PDBU) for 10 or 60 min, were incubated with 1 µg of affinity-purified rabbit polyclonal anti-ERK2 antibody and 30 µl of Protein A/G-agarose at 4 °C for 3 h, immunocomplexes were separated and ERK2 activity was assayed as described in Materials and Methods. In (a), the upper section shows the phosphorylated MBP at 18.5 kDa, as the result of ERK2 activation in the immunocomplexes. MBP phosphorylation as the result of ERK2 activation in PDBU-treated cells is shown as a positive control. In the lower section of panel A the counts per minutes (c.p.m.) of corresponding cut gel lanes are compared. These autoradiographs are representatives of two independent experiments. In panel B, the upper section shows MBP phosphorylation by ERK2 activity in the immunocomplexes obtained from cells treated with ketamine (10 µM) in the presence and absence of cycloheximide (10 µg/ml). In the lower section of (b) the c.p.m. of corresponding cut gel lanes are compared. These autoradiographs are representatives of two independent experiments.

Figure 4. Western blot analysis of smooth muscle cell lysate for phosphorylated ERK2. Aliquots (200 µg protein) of cell lysate, obtained from smooth muscle cells treated with 10 µM ketamine or 1 µM phorbol 12,13-dibutyrate (PDBU) for 10 or 60 min, were incubated with 1 µg of affinity-purified rabbit polyclonal anti-ERK2 antibody and 30 µl of Protein A/G-agarose at 4 °C for 3 h. Immunocomplexes were washed with lysis buffer and resuspended in 2 × Laemmli buffer, boiled and analysed by western analysis using a monoclonal antiphosphotyrosine antibody as described in Materials and Methods. The phosphorylated ERK2 in PDBU-treated cells is shown as a positive control. These autoradiographs are representatives of two independent experiments.

Figure 5. Immunofluorescent light microscopy of ketamine-induced nuclear accumulation of c-fos in smooth muscle cells. Quiescent smooth muscle cells were treated with ketamine (10 μ M) for 1 h. Cells were then fixed and stained with a rabbit polyclonal anti-fos protein as described in Materials and Methods. Fluorescein isothiocyanate-conjugated goat antirabbit IgG was used as a second antibody. (a) shows nuclear accumulation of c-fos in ketamine-treated cells. (b) shows untreated control cells. Magnification $\times$ 400.

signalling mechanisms including activation of MAP kinases and nuclear transcription factors by ketamine. This conclusion is based on the following items of evidence: (1) ketamine enhances DNA synthesis in human aortic smooth muscle cells; (2) ketamine-induced enhancement of DNA synthesis is partially, but significantly, reduced in the presence of a MAP kinase inhibitor; (3) ketamine induces expression, phosphorylation and activation of ERK2 in human smooth muscle cells; (4) ketamine-induced DNA synthesis is dependent on de novo protein synthesis; (5) ketamine-treatment of smooth muscle cells leads to nuclear accumulation of c-fos.

The findings on the enhanced DNA synthesis by ketamine point to its potential as an stimulator of smooth muscle cell proliferation. The findings on ketamine-induced activation of MAP kinases may have important implications. Despite their diversity, virtually all known mitogens act via activation of MAP kinases. The activation of MAP kinases has also been seen by non-mitogenic stimuli (Karin & Hunter 1995, review). In this study, we demonstrated that ketamine induces activation of ERK2. In addition to its involvement in cell proliferation, ERK2, by its kinase activity, exerts substantial regulatory impact on the expression and activity of diverse proteins including enzymes, other kinases, cytoskeletal proteins and transcription factors. Therefore, the ketamine-induced activation of ERK2, in addition to participating in the

signalling mechanisms responsible for the observed enhancement of DNA synthesis, may also play an important regulatory role in other biological processes in smooth muscle cells. Because activation of ERK2 requires an upstream phosphorylation cascade including activation of MAP kinase kinase (MEK) and MEK kinase, future studies need to determine the effects of ketamine on the upstream components of the MAP kinase pathway. Because in this study the activation of MAP kinase pathway is shown to be only partially responsible for the ketamine-induced DNA synthesis, other mechanisms must be involved in this effect of ketamine. This notion is further supported by previous reports demonstrating ketamine-induced effects in smooth muscle cells including the effects of ketamine on Ca^{++} movement in vascular smooth muscle cells (Kanmura *et al.* 1996). Therefore, it will be important to determine the effects of ketamine on other intracellular pathways known to be associated with aortic smooth muscle cell proliferation (Berk *et al.* 1990). Equally important is to determine if the observed effect of ketamine on proliferative response of smooth muscle cells is mediated by a specific receptor or intracellular transporter system. As indicated earlier, factor-stimulated expression and activation of MAP kinases have been demonstrated in smooth muscle cells (Duff *et al.* 1992; Koide *et al.* 1992; Granot *et al.* 1993). Vasopressin causes threonine and tyrosine phosphorylation and activation of MAP kinases in cultured vascular smooth muscle cells (Granot *et al.* 1993). Endothelin-1 stimulates tyrosine phosphorylation and causes activation of ERK1/2 in cultured vascular smooth muscle cells (Koide *et al.* 1992). Whether ketamine exerts its observed effects on MAP kinases directly or this anaesthetic acts via mediators, for example autocrine release of growth factors, is an important question to be addressed in future studies.

In the present study, the results of experiments with cycloheximide which showed that ketamine-induced DNA synthesis, but not ketamine-induced ERK2 activation is dependent on de novo protein synthesis, suggest that the effect of ketamine extends to the intracellular mechanisms downstream to ERK2 activation including those involving the induction of early transcription factors. MAP kinase-induced mitogenic signalling is transmitted into the nucleus where it leads to a cascade of phosphorylation events leading to enhanced transcription of early response genes including c-fos and c-jun. The results of this study do not establish a direct link between ketamine-induced activation of ERK2 and nuclear accumulation of c-fos. They do, however, provide strong evidence in support of the notion that ketamine-induced intracellular mechanisms in human smooth muscle cells indeed transmit into the nucleus and result in activation of nuclear transcription factor c-fos.

In summary, we have established that in human aortic smooth muscle cells ketamine exerts mitogenic effects including stimulation of DNA synthesis, activation of MAP kinases and nuclear accumulation of c-fos. Accordingly, ketamine has the potential to regulate proliferative response in smooth muscle cells. Although the immediate clinical implications of this regulatory effect of ketamine can not be predicted, it is logical to speculate that ketamine-induced effects may alter the response of smooth muscle cells to diverse stimuli activated under pathophysiological conditions including those involved in vascular remodelling and hypertrophy. In the case of patients with coronary artery diseases and those with myocardial infarction and related underlying risk factors, this effect of ketamine may be of clinical relevance.

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