

Effects of Dexmedetomidine on Hippocampal Focal Adhesion Kinase Tyrosine Phosphorylation in Physiologic and Ischemic Conditions

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Background: Dexmedetomidine is a potent and selective α_2 -adrenoceptor agonist that exhibits a broad pattern of actions, including sedation, analgesia, and neuroprotection. Some of these actions (e.g., neuroprotection) may require targets involved in long-term cellular changes. The authors hypothesized that dexmedetomidine increases the expression of active (autophosphorylated) focal adhesion kinase (FAK), a nonreceptor tyrosine kinase playing a pivotal role in cellular plasticity and survival. Therefore, we examined the cellular mechanisms involved in this effect and its sensitivity to oxygen–glucose deprivation (OGD) in rat hippocampal slices.

Methods: The effects of dexmedetomidine on phosphotyrosine³⁹⁷ FAK phosphorylation were studied first with or without various pharmacologic agents in normoxic conditions, and second in a model of pharmacologic preconditioning of slices subjected to 30 min of OGD followed by 1 h of reperfusion. FAK phosphorylation and caspase-3 activation were examined by immunoblotting. Neuronal death was assessed by propidium iodide fluorescence.

Results: Dexmedetomidine produced a dose-related increase in FAK phosphorylation ($187 \pm 4\%$, mean \pm SD, from basal level, $EC_{50} = 0.2 \mu M$; 95% confidence interval, 0.09 – $0.5 \mu M$). This effect was stereoselective and was completely blocked by yohimbine and the combination of the cyclic monophosphate permeant analog 8 bromo cyclic monophosphate and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. It was mimicked by the protein kinase A inhibitor H 89. In contrast, prazosin and the protein kinase C inhibitors chelerythrine and bisindolylmaleimide I were ineffective. OGD induced a significant increase in immunoreactivity of the cleaved caspase-3 17-kd fragment (417 ± 22 ; $P < 0.001$), a decrease in FAK phosphorylation ($78 \pm 12\%$ of control; $P < 0.05$), and production of significant neuronal death. In OGD conditions, a preconditioning application of dexmedetomidine ($0.2 \mu M$, 20-min application, 3 h before anoxia) significantly reduced neuronal death and cleaved caspase-3 expression and significantly attenuated the decrease in phosphorylated FAK content. The dexmedetomidine-induced reduction in caspase-3 expression was significantly decreased by the Src tyrosine kinase inhibitor PP2.

Conclusion: Dexmedetomidine exhibits a preconditioning effect against ischemic injury in hippocampal slices subjected to OGD. Increase in phosphorylation of FAK *via* stimulation of α_2

adrenoceptors and decrease in cleaved caspase-3 expression correlate with dexmedetomidine-induced cell survival.

DEXMEDETOMIDINE is a potent and selective agonist of the α_2 -adrenergic receptors negatively coupled to adenylyl cyclase, which exhibits a fascinating spectrum of actions, including a decrease in heart rate and blood pressure, sedation, anesthetic-sparing effects, and analgesia.^{1,2} Recent reports indicate that dexmedetomidine can be used as a primary sedative in critically ill patients, as well as the sole anesthetic in some surgical patients.^{3,4} Major advances have been made in the understanding of the mechanisms underlying the sedative/anesthetic action of dexmedetomidine. Elegant experiments using a targeted mutation approach in mice support that the α_{2A} -receptor subtype is responsible for its sedative and analgesic effects.⁵ Recent data indicate that dexmedetomidine anesthetic action proceeds *via* activation by α_2 -adrenergic receptors located on locus ceruleus neurons of a specific γ -aminobutyric acid-mediated pathway involved in a particular stage of sleep named non-rotation eyes movies.⁶

Dexmedetomidine exhibits potent neuroprotective effects in various experimental models.^{7,8} However, the cellular effects involved in these actions remain unclear. Recent data support that dexmedetomidine exerts its protective effects *via* a direct effect of the α_2 -adrenergic receptor–adenylyl cyclase pathway on intracellular signaling cascades rather than an indirect effect through a reduction in the release of excitotoxic glutamate or catecholamines.^{8–10} This view is further supported by the demonstration of possible antiapoptotic properties of dexmedetomidine.¹¹ Tyrosine protein kinase proteins, such as the nonreceptor tyrosine kinase focal adhesion kinase (FAK), exert a prominent control on signaling pathways and may couple rapid events, such as action potential or neurotransmitter release, to long-lasting changes in synaptic strength and survival.¹² The neuronal isoform of FAK is activated by various extracellular signals that use a restricted number of intracellular pathways, and the increase in intracellular Ca^{2+} and activation of protein kinase C (PKC) family represents a major intracellular step for stimulating FAK phosphorylation.¹² Several lines of evidence suggest that the neuronal isoform of FAK exhibits antiapoptotic properties. Also, the FAK–Src complex is in tight connection with the Fyn tyrosine kinase, which plays a key role in the activation of extracellular regulated kinase pathway pro-

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moting cell survival. Alternatively, FAK has been reported to induce an antiapoptotic signal *via* the Akt-nuclear factor- κ B and extracellular related kinase pathways.¹²

We have recently demonstrated that the protein expression of active (tyrosine phosphorylated) FAK is sensitive to anesthetic agents except ketamine and that the phospholipase C-PKC pathway is involved in this effect.¹³ Alternatively, FAK tyrosine phosphorylation can also be induced *via* stimulation of receptors (*e.g.*, cannabinoid CB1 receptors) negatively coupled to adenylate cyclase.^{14,15} Therefore, in the current study, we investigated first whether dexmedetomidine affects FAK tyrosine phosphorylation in rat hippocampal slices and the mechanisms involved in this effect. Second, we examined the potential physiologic relevance of these findings in a model of pharmacologic preconditioning of the slices subjected to oxygen-glucose deprivation (OGD).

Materials and Methods

Handling procedures according to the *Guide for the Care and Use of Laboratory Animals* were followed throughout.¹⁶ Experiments were performed on male Sprague-Dawley rats (Iffa-Credo, L'Arbresle, France) weighing 250 g and housed on a 12:12-h light:dark cycle with food and water *ad libitum*. Approval was obtained from the Institutional Animal Care and Use Committee at Paris VII University (Paris, France).

This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France) and the recommendations of the Helsinki Declaration. Therefore, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher (Gressens Pierre, M.D., Ph.D., INSERM U 676, Paris, France).

Experimental Protocol

Animals were killed by stunning (thoracic shock followed by reflex cardiac arrest) and decapitation. Brains were quickly removed; hemispheres were separated until the corpus callosum was observed. The hippocampus of each hemisphere was carefully dissected and incubated in Ca^{2+} -free artificial cerebrospinal fluid (CSF; 4°C; 126.5 mM NaCl, 27.5 mM NaHCO_3 , 2.4 mM KCl, 0.5 mM KH_2PO_4 , 1.93 mM MgCl_2 , 0.5 mM Na_2SO_4 , 4 mM glucose, and 11 mM HEPES adjusted to pH 7.4 with 95%/5% [vol/vol] oxygen-carbon dioxide mixture). Hippocampal slices (300- μm thickness each) prepared with a MacIlwain tissue chopper were transferred to polypropylene tubes (three slices per tube) containing 1 ml artificial CSF (60 min, 37°C). To avoid tyrosine kinases activation at this step of the experiment, Ca^{2+} was omitted from the medium from the dissection phase until the

end of incubation. Slices were incubated for 60 min at 37°C with moderate agitation under a humidified atmosphere of 95%/5% (vol/vol) O_2 - CO_2 until pharmacologic treatments were added together with CaCl_2 and/or an OGD-reperfusion challenge was initiated. Tetrodotoxin (1 μM) was added at the beginning of slice incubation to avoid indirect effects due to neuronal firing.

Slices were transferred to airtight chambers (1 cm^3 volume, 10 slices per chamber) and superfused at 10 ml/min during 30 min with either the same oxygenated or a glucose-free CSF bubbled with 95% nitrogen-5% containing 5 mM dithionite, an oxygen absorbent, as previously described.^{17,18} Temperature, pH, and partial pressure of oxygen (PO_2) and carbon dioxide (PCO_2) were closely monitored. Temperature was servo-controlled to 37°C. After the period of simulated ischemia, slices were recovered in oxygenated buffered CSF for 1 h. The sequence of 30 min of ischemia followed by 1 h was selected according to a preliminary "time-response" study indicating that it provided optimal changes in the content of both FAK and activated caspase 3.

In normoxic normoglycemic conditions, dexmedetomidine and levomedetomidine were applied at various concentrations (10^{-8} M to 10^{-3} M) for 5 min. This duration of incubation was determined according to preliminary experiments in which FAK phosphorylation induced by 1 μM dexmedetomidine was plotted against duration of incubation (1, 2, 5, 10, 20, and 30 min) in the presence of the phosphatase inhibitor orthovanadate (1 mM). It corresponded to the point for which the ceiling was reached after a linear increase in phosphorylation between 1 and 5 min. Other pharmacologic agents supposed to increase FAK phosphorylation were applied for 5 min as well. In anoxic-aglycemic conditions, dexmedetomidine was applied as a preconditioning stimulus during 20 min,¹⁸ and then oxygenation and buffering of the slices were continued for 3 h before the 30-min period of ischemia was induced. This was followed by a 1-h period of reperfusion with oxygen glucose containing CSF. Agents supposed to block the dexmedetomidine (or any other activator)-induced changes in FAK phosphorylation and/or cleaved caspase-3 expression were administered 1 h before dexmedetomidine.

At the end of the experiments, CSF was aspirated, and slices were frozen in liquid nitrogen, homogenized by sonication in 200 μl of a solution of 1% (wt/vol) sodium dodecyl sulfate, 1 mM sodium orthovanadate, and anti-proteases (50 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 5 $\mu\text{g}/\text{ml}$ pepstatin) in water at 100°C, and placed in a boiling bath for 5 min. Homogenates were stored at -80°C until processing. To investigate the putative role of phosphatases in the changes observed in FAK phosphorylation, orthovanadate, a tyrosine phosphatase inhibitor, and okadaic acid, a serine-threonine phosphatase inhibitor, were administered at active concentrations (10^{-3} M

and 10^{-6} M, respectively) from the beginning of incubation in physiologic and ischemic conditions.

Chemicals

The following agents were studied alone or in combination: dexmedetomidine and levomedetomidine (10^{-8} M to 10^{-3} M), tetrodotoxin ($1\text{ }\mu\text{M}$; Sigma, St-Quentin Fallavier, France), the inhibitors of PKC chelerythrine (10^{-5} M; Sigma) and bisindolylmaleimide I (10^{-5} M; Sigma), phorbol 12-myristate 13-acetate (PMA, $0.1\text{ }\mu\text{M}$; Sigma), the analog of cyclic adenosine monophosphate (cAMP) 8 bromo cAMP (4×10^{-3} M; Sigma), the inhibitor of phosphor-diesterases 3-isobutyl-1-methylxanthine (10^{-4} M; Sigma), methoxamine (10^{-5} M; Sigma), the inhibitor of cAMP-dependent protein kinase H 89 (10^{-5} M; Sigma), prazosin (10^{-4} M; Sigma), yohimbine (10^{-4} M; Sigma), the inhibitor of Src tyrosine kinase 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, 10^{-5} M; Calbiochem, Nottingham, United Kingdom), and the phosphatases inhibitors sodium orthovanadate (1 mM ; Sigma) and okadaic acid (10^{-6} M; Sigma).

Immunoblot Analysis

Protein concentration in the homogenates was determined with a bicinchoninic acid-based method, using bovine serum albumin as the standard. Equal amounts of protein ($30\text{ }\mu\text{g}$) were subjected to 6% (wt/vol) polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and transferred electrophoretically to nitrocellulose. For detection of phosphorylated FAK, immunoblot analysis was performed with affinity-purified rabbit antiphosphotyrosine antibodies (monoclonal mouse immunoglobulin [Ig] G, clone 4G10; Euromedex 05-321, Souffelweysheim, France). Primary antibodies were labeled with peroxidase-coupled antibodies against rabbit IgG, which were detected by exposure of molecular probe autoradiographic films in the presence of a chemiluminescent reagent (ECL; Amersham, Little Chalfont, United Kingdom). The specificity of the immunoreactivity for FAK was assessed by its competition in the presence of $50\text{ }\mu\text{M}$ O-phosphotyrosine. Variations between gels were controlled by expressing the results as a percentage of increase or decrease from control of FAK normalized to β -actin. For each band, blank values were subtracted before calculating the ratio.

Identification of phosphorylated FAK was performed with a rabbit anti-tyrosine³⁹⁷ FAK phosphospecific antibody (Biosource International, Camarillo, CA; diluted 1:1,000) after pooling five to eight independent samples. Identification of total FAK was performed using an anti-FAK antibody directed against the nonphosphorylated

residues of the protein (monoclonal mouse IgG, clone 4-47; Euromedex 05-537, Souffelweysheim, France). Immunoreactive bands were quantified using a computer-assisted densitometer and normalized to β -actin expression (quantified by using the specific monoclonal antiactin A5316 antibody Sigma) ratio (Cohu High Performance charge-coupled device camera; Gel Analyst 3.01 pci, Paris, France). The validity of the actin control in both physiologic and ischemic conditions was tested by comparing the normalization of data to either actin or total FAK in both physiologic and conditions and OGD.

Quantification of Cell Death

Quantification of cell death was performed using two methods: detection of active cleaved caspase 3 by Western blot analysis and quantification of cell death using propidium iodide (PI) fluorescence.

Detection of active cleaved caspase 3 was performed using Western blot analysis (see Immunoblot Analysis) using a rabbit polyclonal IgG anti-caspase 3-specific antibodies detecting both the 32-kd entire protein and the 17-kd fragment produced by cleavage of caspase 3 when activated (Upstate biotechnology, diluted 1:2,000; Euromedex). The 17-kd band was considered cleaved caspase 3 and was taken into consideration in the statistical analysis.

Quantification of cell death and dexmedetomidine neuroprotection was also detected using fluorescent PI (Invitrogen, Cergy-Pontoise, France, P-3566).^{19,20} PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per four to five base pairs of DNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the maximal absorption wave for PI being 535 nm and maximal fluorescence emission wave being 617 nm. PI was added to the artificial CSF ($2\text{ }\mu\text{g/ml}$) in three independent experiments. In each of them, five slices were randomly selected and processed for serial $20\text{-}\mu\text{m}$ coronal sections cut along the entire hippocampus. Stained cells were examined using a fluorescence microscope equipped with an appropriate filter (UV-2A; Zeiss, Oberkochen, Germany; excitation, 530 nm; emission, $> 600\text{ nm}$), and images were digitalized. All the slices were analyzed the same time by an observer unaware of treatment assignment. For each experiment, PI fluorescence from five areas of the CA1 subfield were analyzed using Image J 1.31v software.||

Statistical Analysis

The data presented were collected from five to eight independent experiments run in triplicate. Concentration-response curves were generated using GraphPad 4.0 software (Intuitive Software for Science, San Diego, CA). Statistical analysis was performed using Statistica 6.0 (Statsoft, Inc., Tulsa, OK). All of the software programs were run on a Presario 2100 (Hewlett-Packard,

|| National Institutes of Health. Available at: [www://rsb.info.nih.gov/ij/](http://www.rsb.info.nih.gov/ij/). Accessed May 17, 2005.

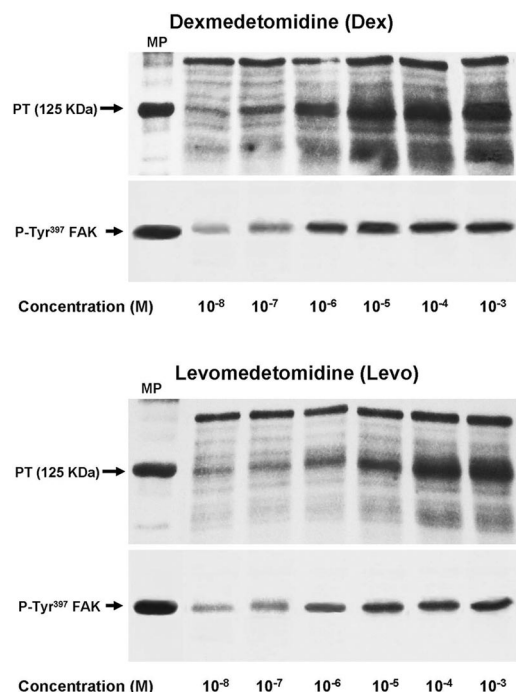


Fig. 1. Typical example of a Western blot showing the effects of dexmedetomidine on total tyrosine phosphorylation (PT) and specific phospho-tyrosine³⁹⁷ focal adhesion kinase (P-Tyr³⁹⁷ FAK) phosphorylation. MP = molecular probe.

Houston, TX) COMPAQ computer. For tyrosine phosphorylation or caspase-3 protein expression (15–24 experiments per condition), normality of distributions was first assessed by the Fisher exact test for equality of variances. Statistical analysis was then performed by analysis of variance with the Scheffé *post hoc* correction for multiple comparisons.

Results (mean \pm SD) are expressed as a percentage of control FAK or caspase-3 protein expression. $P < 0.05$ was considered the threshold for significance.

Results

Experiments Performed in Physiologic Conditions

Both dexmedetomidine and levomedetomidine produced a significant, concentration-related, similar increase in phosphotyrosine immunoreactivity of both the 125-kd band and the specific FAK one (figs. 1 and 2). However, the EC_{50} values (maximal effect) were significantly different for dexmedetomidine ($0.22 \mu\text{M}$ [95% confidence interval, 0.09 – $0.49 \mu\text{M}$]; $187 \pm 6\%$) and levomedetomidine ($12.5 \mu\text{M}$ [95% confidence interval, 2.2 – $17.8 \mu\text{M}$]; $186 \pm 9\%$) ($P < 0.05$; fig. 2). In contrast, dexmedetomidine did not significantly increase immunoreactivity of total FAK (data not shown). Omitting tetrodotoxin from the incubation medium did not significantly change tyrosine phosphorylation (data not shown). For each dexmedetomidine concentration tested, no significant difference between tyrosine phosphorylation normalized to either total FAK or actin was

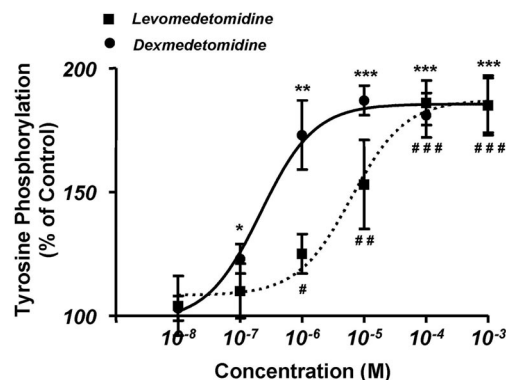


Fig. 2. Concentration–response curves of the effects of dexmedetomidine (10^{-8} to 10^{-3} M) and levomedetomidine (10^{-8} to 10^{-3} M) on tyrosine phosphorylation in rat hippocampal slices. Data (mean \pm SD) are expressed as a fractional tyrosine phosphorylation on actin ratio increase from basal phosphorylation (control: 100%). * $P < 0.05$ versus control, ** $P < 0.01$ versus control, *** $P < 0.001$ versus control for dexmedetomidine. # $P < 0.05$ versus control, ## $P < 0.01$ versus control, ### $P < 0.001$ versus control for levomedetomidine.

present (data not shown). In addition, the EC_{50} s of dexmedetomidine-induced FAK phosphorylation normalized to either total FAK or actin were not significantly different: $0.13 \mu\text{M}$ (95% confidence interval, 0.05 – $0.30 \mu\text{M}$) and $0.22 \mu\text{M}$ (95% confidence interval, 0.09 – $0.49 \mu\text{M}$) (data not shown).

The increase in FAK phosphorylation induced by dexmedetomidine ($0.2 \mu\text{M}$) was completely blocked by the α_2 -adrenoceptor antagonist yohimbine (10^{-5} M) and the association of the permeant cAMP analog 8 bromo cAMP (4×10^{-3} M) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (10^{-4} M) (fig. 3). Further, dexmedetomidine effect on FAK phosphorylation was

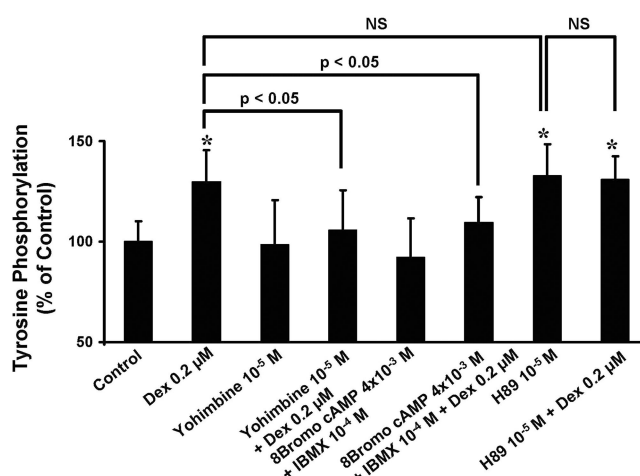
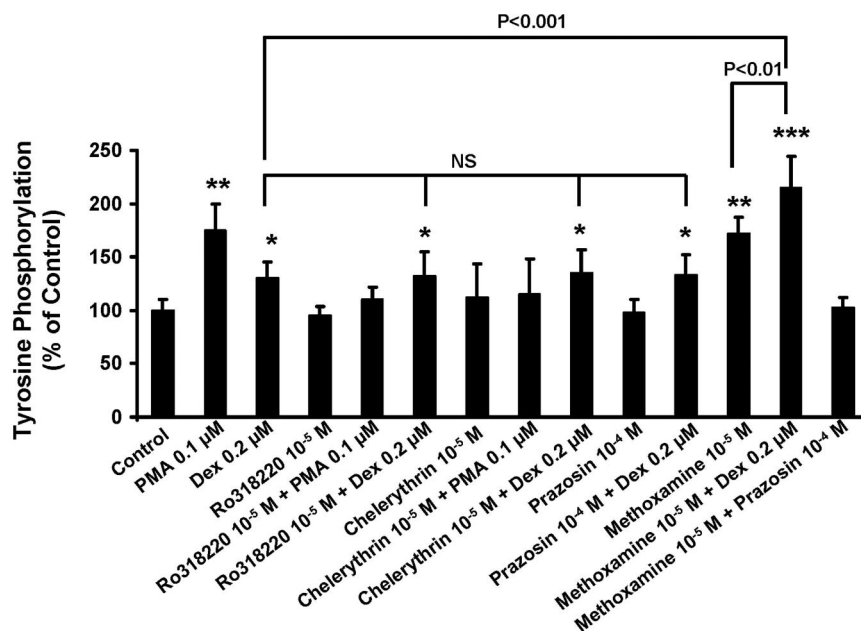


Fig. 3. Effects of yohimbine (10^{-5} M), H 89 (10^{-4} M), and association of 8 bromo cyclic adenosine monophosphate (8 Bromo cAMP, 4×10^{-3} M) and 3-isobutyl-1-methylxanthine (IBMX, 10^{-4} M) on dexmedetomidine (Dex, $0.2 \mu\text{M}$)-induced focal adhesion kinase phosphorylation in rat hippocampal slices. Data (mean \pm SD) are expressed as a fractional 125-kd tyrosine phosphorylation on actin ratio increase from basal phosphorylation (control: 100%). * $P < 0.05$ versus control. NS = not significant.

Fig. 4. Effects of prazosin (10^{-4} M), methoxamine (10^{-5} M), chelerythrine (10^{-5} M), bisindolylmaleimide I (Ro318220, 10^{-5} M), and phorbol 12-myristate 13-acetate (PMA, $0.1 \mu\text{M}$) on dexmedetomidine (Dex, $0.2 \mu\text{M}$)-induced focal adhesion kinase phosphorylation in rat hippocampal slices. Data (mean \pm SD) are expressed as a fractional 125-kd tyrosine phosphorylation on actin ratio increase from basal phosphorylation (control: 100%). * $P < 0.05$ versus control, ** $P < 0.01$ versus control, *** $P < 0.001$ versus control. NS = not significant.



mimicked by H 89, but no additivity of these effects was detected (fig. 3). In contrast, the α_1 -adrenoceptor antagonist prazosin (10^{-4} M) did not affect dexmedetomidine-induced increase in FAK phosphorylation, whereas it completely blocked the effect of methoxamine (10^{-5} M) (fig. 4). The structurally distinct inhibitors of PKC, chelerythrine (10^{-5} M) and bisindolylmaleimide I (10^{-5} M), did not affect dexmedetomidine effect on FAK phosphorylation, whereas they blocked the effect of the PKC activator phorbol 12-myristate 13-acetate ($0.1 \mu\text{M}$). The combination of dexmedetomidine ($0.2 \mu\text{M}$) and methoxamine (10^{-5} M) produced a significantly additive effect on FAK phosphorylation (fig. 4).

Experiments Performed in Oxygen-Glucose-deprived Slices

Po_2 , Pco_2 , and pH in the anoxic-aglycemic versus control chambers were the following: 4 ± 2 versus 450 ± 21 mmHg ($P < 0.05$), 40 ± 3 versus 41 ± 3 mmHg (not significant), and 7.4 ± 0.02 versus 7.4 ± 0.03 (not significant). A 30-min period of OGD followed by 1 h of reperfusion resulted in both a marked decrease in phosphorylated FAK content ($78 \pm 12\%$ of normoxic normoglycemic control; $P < 0.05$) and a significant increase in the 17-kd fragment of caspase-3 immunoreactivity (437 ± 22 ; $P < 0.001$; figs. 5A and B). At this time, the intensity of PI fluorescence was significantly increased in comparison with physiologic conditions ($5,721 \pm 204$ vs. 27 ± 1.6 ; $P < 0.001$; fig. 6). Application of dexmedetomidine ($0.2 \mu\text{M}$) during 20 min, 3 h before the anoxic aglycemic challenge, significantly attenuated neuronal death ($5,721 \pm 204$ vs. $2,896 \pm 255$; $P < 0.001$; fig. 6) as well as the decrease in phosphorylated FAK content. Also, it significantly reduced 17-kd caspase-3 fragment immunoreactivity. In contrast, coapplication of

the Src tyrosine kinase inhibitor PP2 (10^{-5} M) together with dexmedetomidine ($0.2 \mu\text{M}$) significantly, but not totally, reduced the decrease in 17-kd caspase-3 fragment of content (figs. 5A and B). As was the case in physiologic conditions, normalization of data to either actin or total FAK was not significantly different in any case (data not shown). The presence of orthovanadate or okadaic acid from the onset of incubation did not induce any change in FAK phosphorylation or cleaved caspase-3 expression (figs. 7A and B).

Discussion

We have shown that clinically relevant concentrations of dexmedetomidine increase phosphorylation of FAK tyrosine kinase in rat hippocampal slices. This effect is mediated *via* activation of the α_2 -adrenoceptor-adenylate cyclase pathway. We also provide evidence that a preconditioning application of dexmedetomidine attenuates both the decrease in FAK tyrosine phosphorylation and the increase in cleaved caspase-3 expression observed in response to OGD. These effects correlate with a neuroprotective effect of dexmedetomidine observed in the slices.

Methodologic Considerations

Control of Po_2 , Pco_2 , and pH in the superfusion chambers was satisfactory, because Po_2 of less than 5 mmHg was obtained in the anoxic chambers. Dexmedetomidine induced a concentration-related increase in FAK tyrosine phosphorylation. The specificity of the antityrosine³⁹⁷ FAK antibody for the phosphorylated (active) form of FAK has been demonstrated previously.^{12,14,21} This represents the first step of FAK activation and oc-

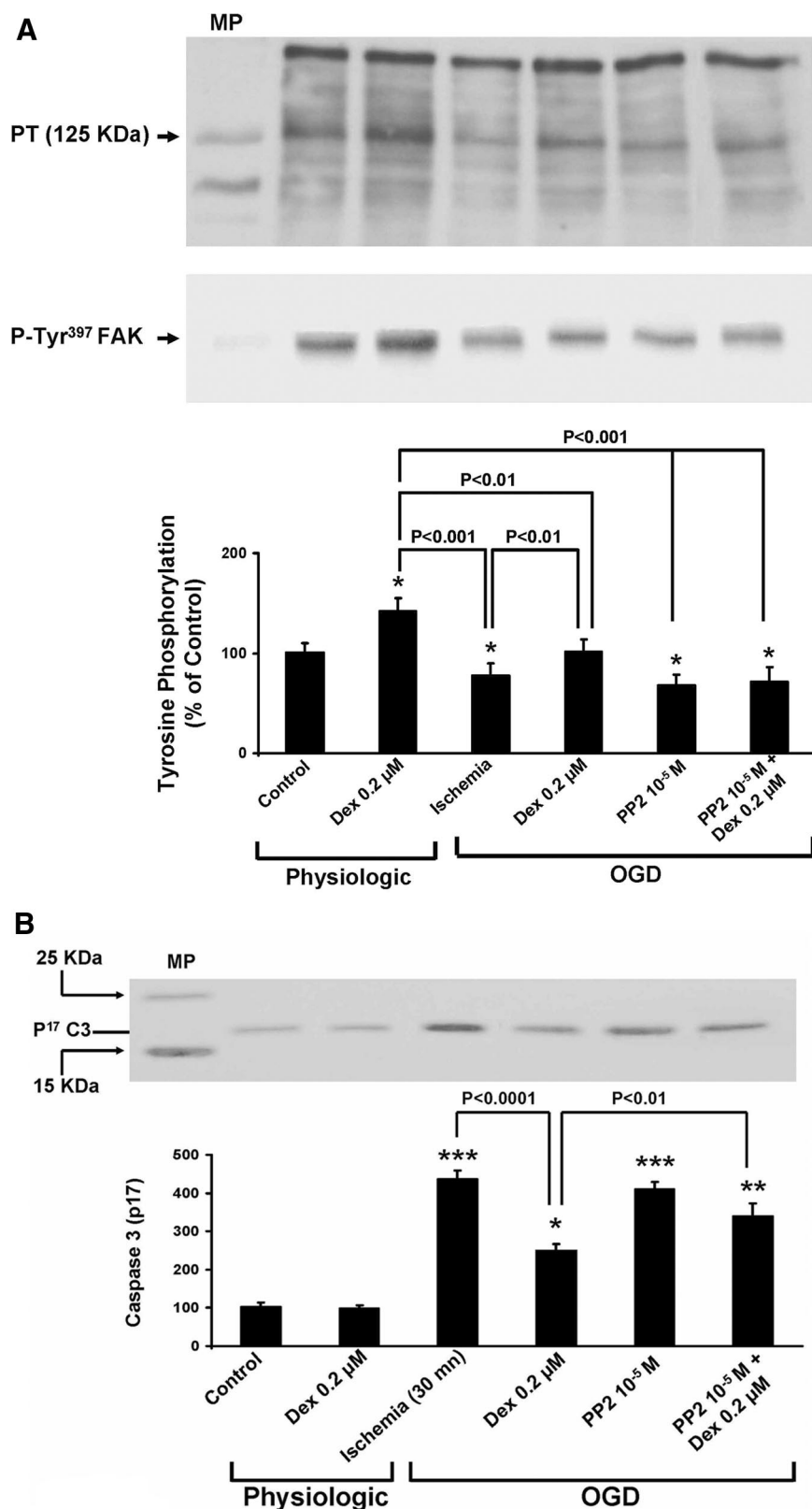


Fig. 5. (A) Effects of dexmedetomidine (Dex, 0.2 μ M), PP2 (10⁻⁵ M), and the association of dexmedetomidine and PP2 in presence or absence of orthovanadate (1 mM) during all the procedure in physiologic (normal cerebrospinal fluid [CSF] superfusion during all the procedure) or oxygen–glucose deprivation (OGD) (3 h normal CSF superfusion, 30 min oxygen- and glucose-free CSF superfusion, and 1 h normal CSF superfusion) conditions on focal adhesion kinase phosphorylation. Data (mean \pm SD) are expressed as a fractional 125-kd tyrosine phosphorylation on actin ratio increase from basal phosphorylation for physiologic condition (control physiologic: 100%). Western blot bands show total 125-kd tyrosine phosphorylation (PT, 125-kd; upper panels) and phospho-tyrosine³⁹⁷ focal adhesion kinase (P-Tyr³⁹⁷ FAK; lower panels). (B) Effects of dexmedetomidine (0.2 μ M), PP2 (10⁻⁵ M), and the association of dexmedetomidine and PP2, in presence or absence of orthovanadate (1 mM) during all the procedure, in physiologic (normal CSF superfusion during all the procedure) or OGD (3 h normal CSF superfusion, 30 min oxygen- and glucose-free CSF superfusion, and 1 h normal CSF superfusion) conditions on P¹⁷ caspase-3 expression. Data (mean \pm SD) are expressed as a fractional P¹⁷ caspase-3 (P¹⁷ C3) increase from basal for physiologic condition (control physiologic: 100%). Western blot bands show P¹⁷ caspase 3. MP = molecular probe.

curs by autophosphorylation of the kinase on its tyrosine³⁹⁷ residue.¹² Our data indicate also that phosphatases were very likely to not play a major role in the reported effects. Also, it was of particular importance to

show first that dexmedetomidine actually increased the content of phosphorylated FAK, and not simply the total amount of FAK, and second that the expression of results as normalized to actin concentrations was not af-

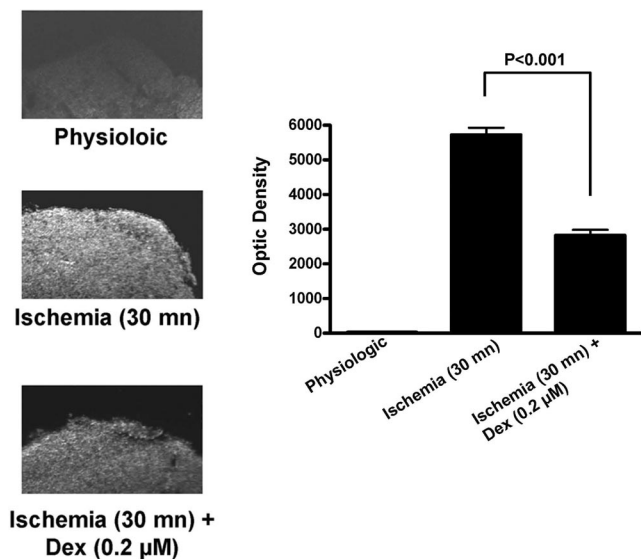


Fig. 6. (Left) Propidium iodide fluorescence on CA1 hippocampal areas ($\times 20$ magnification). (Right) Propidium iodide fluorescence in physiologic (normal cerebrospinal fluid superfusion during all the procedure) or ischemia (3 h normal cerebrospinal fluid superfusion, 30 min oxygen- and glucose-free cerebrospinal fluid superfusion, and 1 h normal cerebrospinal fluid superfusion) experiments in presence or absence of dexmedetomidine preconditioning. * $P < 0.001$.

ected by the possible degradation of FAK, but not actin, under OGD. We therefore compared a second standard, *i.e.*, total FAK content, which includes both nonphosphorylated and phosphorylated FAK, to actin content to normalize the data. Our results indicate first that dexmedetomidine actually increased the phosphorylated FAK content, and second that normalization to actin was valid under both physiologic and ischemic conditions.

Dexmedetomidine Increases FAK Phosphorylation via Activation of α_2 -Adrenoceptors

The effect of dexmedetomidine on FAK phosphorylation was likely not to be mediated *via* α_1 adrenoceptors. We have previously shown that most of the intravenous and volatile anesthetics increased FAK phosphorylation *via* activation of the phospholipase C-PKC pathway and that this effect was not shared by the nonimmobilizer F6.¹³ The lack of effect of the structurally distinct PKC antagonists bisindolylmaleimide I and chelerythrine in our study suggests that dexmedetomidine effects on FAK phosphorylation are not mediated by PKC. The pharmacologic approach we used here strongly supports that dexmedetomidine increases FAK phosphorylation by stimulation of α_2 adrenoceptors. However, the receptor subtype (α_{2A} or α_{2C}) involved in this effect was not determined here. Our results are consistent with the previous observation that decreasing adenylate cyclase activity (*e.g.*, *via* activation of the cannabinoid CB1 receptors) results in an increase in FAK phosphorylation.^{14,15,22} However, the relations between reduced cAMP and protein kinase A activity and FAK phosphor-

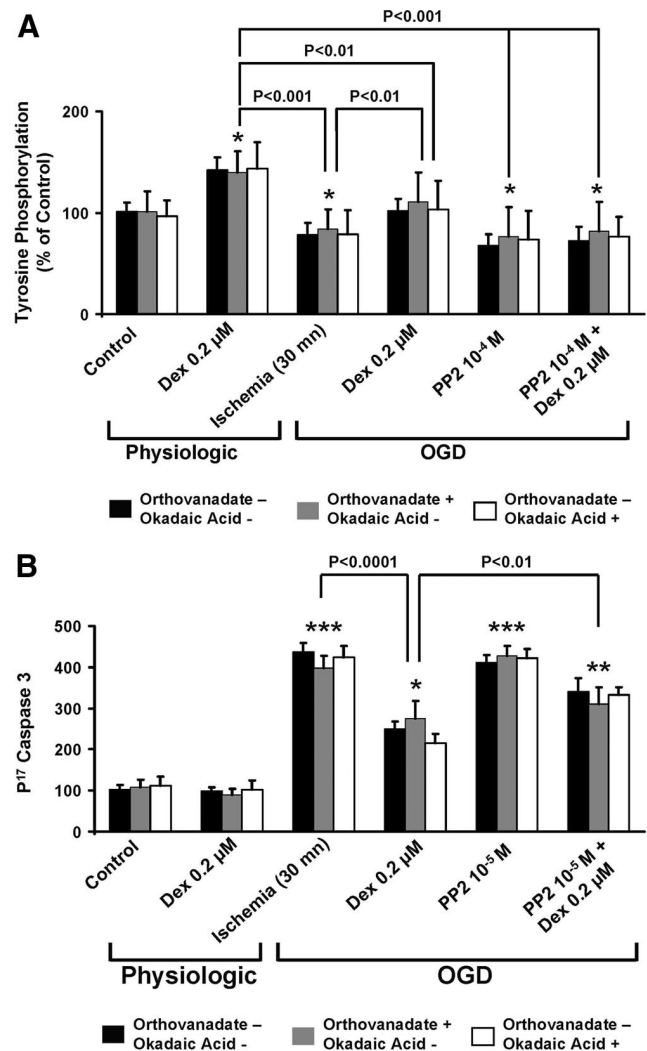


Fig. 7. (A) Effects of dexmedetomidine (Dex, $0.2 \mu\text{M}$), PP2 (10^{-5} M), and the association of dexmedetomidine and PP2 in physiologic (normal cerebrospinal fluid [CSF] superfusion during all the procedure) or oxygen-glucose deprivation (OGD) (3 h normal CSF superfusion, 30 min oxygen- and glucose-free CSF superfusion, and 1 h normal CSF superfusion) conditions with or without the phosphatases inhibitors orthovanadate (1 mM) or okadaic acid (10^{-5} M) on focal adhesion kinase phosphorylation. Data (mean \pm SD) are expressed as a fractional 125-kD tyrosine phosphorylation on actin ratio increase from basal phosphorylation for physiologic condition (control physiologic: 100%) and OGD condition (control OGD: 100%). * $P < 0.05$ versus control, ** $P < 0.01$ versus control, *** $P < 0.001$ versus control. (B) Effects of dexmedetomidine ($0.2 \mu\text{M}$), PP2 (10^{-5} M), and the association of dexmedetomidine and PP2 in physiologic (normal CSF superfusion during all the procedure) or OGD (3 h normal CSF superfusion, 30 min oxygen- and glucose-free CSF superfusion, and 1 h normal CSF superfusion) conditions with or without the phosphatases inhibitors orthovanadate (1 mM) or okadaic acid (10^{-6} M) on P17 caspase 3. Data (mean \pm SD) are expressed as a fractional P17 caspase-3 increase from basal immunoreactivity for physiologic condition (control physiologic: 100%) and OGD condition (control OGD: 100%). * $P < 0.05$ versus control, ** $P < 0.01$ versus control, *** $P < 0.001$ versus control.

ylation remain to be clarified. In some experimental systems, such as fibroblasts or endothelial cell cultures, the role of SHP2, a tyrosine phosphatase controlling FAK phosphorylation, has been suggested.^{23,24} These obser-

vations are not supported by our data showing insensitivity of FAK phosphorylation to phosphatase inhibitors. In differentiated tissue, such as hippocampal slices from adult rats, changes in cAMP concentrations induced by a Gi-coupled receptor or pharmacologic manipulation of protein kinase A activity seem sufficient to activate a signaling pathway involving FAK, similar to that triggered by integrin-dependant cell adhesion.¹² The effect of dexmedetomidine on FAK phosphorylation exhibited a significant degree of stereoselectivity, because the EC₅₀ value observed for FAK phosphorylation by the levogyre medetomidine isomer (levomedetomidine) was significantly greater than that of dexmedetomidine. Interestingly, levomedetomidine has a significantly lower anesthetic potency in various species, which supports physiologic relevance of the present findings.^{25,26}

Dexmedetomidine Exhibits a Preconditioning Effect in an In Vitro Model of Neuroprotection

Consistent with our previous findings, A 30-min period of OGD resulted in a marked increase in the intensity of PI fluorescence.²⁷ This correlated with a decrease in the content of phosphorylated FAK in the slices. This is most likely to be explained by the energy deprivation caused by OGD, because the phosphorylation process is adenosine triphosphate dependent,^{28,29} or by activation of phosphatase.³⁰ Our results do not support phosphatase stimulation because no difference in FAK phosphorylation or caspase activation was found when both tyrosine phosphatases and serine-threonine phosphatases were inhibited. Energy deprivation induces in hippocampal slices a cascade of events triggered by excitotoxic glutamate and leading to neuronal death by necrosis, apoptosis, or both.²⁸ The increase in the expression of the 17-kd fragment of caspase 3, a key enzyme in the production of apoptotic death, suggests that caspase 3 has been cleaved and activated by the OGD. Brain caspase-3 expression has been found increased when ischemia or hypoperfusion is present.^{31,32} Therefore, apoptotic cell death was likely to be present in the slices subjected to OGD and reperfusion.

Reduction of cell death by dexmedetomidine correlated with restoration of the content of phosphorylated FAK to physiologic levels. Expression of cleaved caspase 3 was significantly, but not totally, reduced by dexmedetomidine (0.2 μ M) under OGD. This is consistent with results previously obtained by Engelhard *et al.*¹¹ showing that dexmedetomidine enhances the expression of regulatory proteins favoring antiapoptosis and decreases that of proapoptotic ones in rat brains subjected to 30 min of focal ischemia.¹³ Further, we provide evidence for the first time that dexmedetomidine exerts preconditioning effects toward brain ischemic injury. Activation of the Src-family kinases by FAK autophosphorylation represents a critical step to allow phosphorylation of tyrosine residues in the catalytic and carboxyterminal

domain of FAK.³³ These residues bind target molecules such as extracellular related kinase and induce their activation.¹² We found that the effect of dexmedetomidine on caspase 3 expression in ischemic conditions was significantly reduced by the Src tyrosine kinase inhibitor PP2. This is consistent with previous data showing that adenylate cyclase-mediated cannabinoid-induced increase in FAK-related kinases, such as Fyn, was sensitive to PP2.²⁸ Because reduction of 17-kd caspase 3 elicited by PP2 was only partial (fig. 5B), it is likely that other mechanisms, such as reduction in glutamate release, are involved in dexmedetomidine-induced neuroprotection. Indeed, reduction in glutamate transmission is expected to decrease FAK phosphorylation and can therefore be considered as a potential additional mechanism of dexmedetomidine induced neuroprotection. Altogether, these experiments support that preservation of FAK phosphorylation and inhibition of caspase-3 activation contribute, at least in part, to the neuroprotective effects of dexmedetomidine observed in our model.

Physiologic Relevance

The interpretation of the current findings might have some physiologic relevance but also exhibits some limitations. First, caution should always be used when extrapolating *in vitro* data to the clinical setting. Second, our model allows detecting only short-term protective actions of dexmedetomidine *in vitro*. It cannot be excluded that the protective effects of dexmedetomidine reported here are no longer present after days or weeks.³⁴ However, our results and data from other groups support that the protective effects of dexmedetomidine against ischemic injury proceed in part *via* anti-apoptotic mechanisms.¹¹ Third, whether dexmedetomidine acts at the pretranscriptional or posttranscriptional level to increase FAK phosphorylation cannot be derived from our data. However, the efficient dexmedetomidine concentrations reported here are low and fairly consistent with those that can be anticipated to be found in the brain of rats protected by this agent against ischemic injury^{8,10} or in the plasma of humans treated with this agent.^{35,36}

In summary, dexmedetomidine exhibits a preconditioning effect against ischemic injury in the hippocampal slice subjected to OGD. Increase in phosphorylation of FAK *via* stimulation of α_2 adrenoceptors and decrease of cleaved caspase-3 expression correlate with dexmedetomidine-induced cell survival.

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References

1. Fraser CM, Arakawa S, McCombie WR, Venter JC: Cloning, sequence analysis, and permanent expression of a human α_2 -adrenergic receptor in

Chinese hamster ovary cells: Evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. *J Biol Chem* 1989; 264:11754-61

2. Kamibayashi T, Maze M: Clinical uses of α_2 -adrenergic agonists. *ANESTHESIOLOGY* 2000; 93:1345-9

3. Martin E, Ramsay G, Mantz J, Sum-Ping ST: The role of the α_2 -adrenoceptor agonist dexmedetomidine in postsurgical sedation in the intensive care unit. *J Intensive Care Med* 2003; 18:29-41

4. Ramsay MA, Luterma DL: Dexmedetomidine as a total intravenous anesthetic agent. *ANESTHESIOLOGY* 2004; 101:787-90

5. Lakhani PP, MacMillan LB, Guo TZ, McCool BA, Lovinger DM, Maze M, Limbird LE: Substitution of a mutant α_2 -adrenergic receptor via "hit and run" gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. *Proc Natl Acad Sci U S A* 1997; 94:9950-5

6. Nelson LE, Lu J, Guo T, Saper CB, Franks NP, Maze M: The α_2 -adrenoceptor agonist dexmedetomidine converges on an endogenous sleep-promoting pathway to exert its sedative effects. *ANESTHESIOLOGY* 2003; 98:428-36

7. Hoffman WE, Kochs E, Werner C, Thomas C, Albrecht RF: Dexmedetomidine improves neurologic outcome from incomplete ischemia in the rat: Reversal by the α_2 -adrenergic antagonist atipamezole. *ANESTHESIOLOGY* 1991; 75:328-32

8. Laudenbach V, Mantz J, Lagercrantz H, Desmonts JM, Evrard P, Gressens P: Effects of α_2 -adrenoceptor agonists on perinatal excitotoxic brain injury: Comparison of clonidine and dexmedetomidine. *ANESTHESIOLOGY* 1996; 104:134-41

9. Kim HK, Zornow MH, Strnat MA, Maze M: Dexmedetomidine does not attenuate increases in excitatory amino acids after transient global ischemia in the rabbit. *J Neurosurg Anesthesiol* 1996; 8:230-6

10. Engelhard K, Werner C, Kaspar S, Mollenberg O, Blobner M, Bachl M, Kochs E: Effect of the α_2 -agonist dexmedetomidine on cerebral neurotransmitter concentrations during cerebral ischemia in rats. *ANESTHESIOLOGY* 2002; 96:450-7

11. Engelhard K, Werner C, Eberspacher E, Bachl M, Blobner M, Hildt E, Hutzler P, Kochs E: The effect of the α_2 -agonist dexmedetomidine and the N-methyl-D-aspartate antagonist S(+)-ketamine on the expression of apoptosis-regulating proteins after incomplete cerebral ischemia and reperfusion in rats. *Anesth Analg* 2003; 96:524-31

12. Girault JA, Costa A, Derkinderen P, Studler JM, Toutant M: FAK and PYK2/CAKbeta in the nervous system: A link between neuronal activity, plasticity and survival? *Trends Neurosci* 1999; 22:257-63

13. Derkinderen P, Toutant M, Burgaya F, Le Bert M, Siciliano JC, de Franciscis V, Gelman M, Girault JA: Regulation of a neuronal form of focal adhesion kinase by anandamide. *Science* 1996; 273:1719-22

14. Dahmani S, Tesnière A, Rouelle D, Toutant M, Desmonts JM, Mantz J: Effects of anesthetic agents on focal adhesion kinase (pp125FAK) tyrosine phosphorylation in rat hippocampal slices. *ANESTHESIOLOGY* 2004; 101:344-53

15. Zhou D, Song ZH: CB1 cannabinoid receptor-mediated tyrosine phosphorylation of focal adhesion kinase-related non-kinase. *FEBS Lett* 2002; 525:164-8

16. National Institutes of Health: Guide for the Care and Use of Laboratory Animals. NIH Publication No. 86-23. Bethesda, National Institutes of Health, 1996, pp 58-9

17. Popovic R, Liniger R, Bickler PE: Anesthetics and mild hypothermia similarly prevent hippocampal neuron death in an *in vitro* model of cerebral ischemia. *ANESTHESIOLOGY* 2000; 92:1343-9

18. Yuan HB, Huang Y, Zheng S, Zuo Z: Hypothermic preconditioning increases survival of Purkinje neurons in rat cerebellar slices after an *in vitro* simulated ischemia. *ANESTHESIOLOGY* 2004; 100:331-7

19. Laake JH, Haug FM, Wieloch T, Ottersen OP: A simple in vitro model of

ischemia based on hippocampal slice cultures and propidium iodide fluorescence. *Brain Res Brain Res Protoc* 1999; 4:173-84

20. Norberg J, Kristensen BW, Zimmer J: Markers for neuronal degeneration in organotypic slice cultures. *Brain Res Brain Res Protoc* 1999; 3:278-90

21. Derkinderen P, Siciliano J, Toutant M, Girault JA: Differential regulation of FAK+ and PYK2/Cakbeta, two related tyrosine kinases, in rat hippocampal slices: Effects of LPA, carbachol, depolarization and hyperosmolarity. *Eur J Neurosci* 1998; 10:1667-75

22. Derkinderen P, Toutant M, Kadare G, Ledent C, Parmentier M, Girault JA: Dual role of Fyn in the regulation of FAK+6,7 by cannabinoids in hippocampus. *J Biol Chem* 2001; 276:38289-96

23. Yu DH, Qu CK, Henegariu O, Lu X, Feng GS: Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J Biol Chem* 1998; 273:21125-31

24. Manes S, Mira E, Gomez-Mouton C, Zhao ZJ, Lacalle RA, Martinez-AC: Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Mol Cell Biol* 1999; 19:3125-35

25. Segal IS, Vickery RG, Walton JK, Doze VA, Maze M: Dexmedetomidine diminishes halothane anesthetic requirements in rats through a postsynaptic α_2 adrenergic receptor. *ANESTHESIOLOGY* 1988; 69:818-23

26. Tonner PH, Scholz J, Koch C, Schulte am Esch J: The anesthetic effect of dexmedetomidine does not adhere to the Meyer-Overton rule but is reversed by hydrostatic pressure. *Anesth Analg* 1997; 84:618-22

27. Dahmani S, Tesnière A, Rouelle D, Desmonts JM, Mantz J: Thiopental and isoflurane attenuate the decrease in hippocampal phosphorylated Focal Adhesion Kinase (pp 125 FAK) content induced by oxygen glucose deprivation. *Br J Anaesth* 2004; 93:270-4

28. Larsen M, Haugstad TS, Berg-Johnsen J, Langmoen IA: The effect of isoflurane on brain amino acid release and tissue content induced by energy deprivation. *J Neurosurg Anesthesiol* 1998; 10:166-70

29. Kass IS, Amorim P, Chambers G, Austin D, Cottrell JE: The effect of isoflurane on biochemical changes during and electrophysiological recovery after anoxia in rat hippocampal slices. *J Neurosurg Anesthesiol* 1997; 9:280-6

30. Horiguchi T, Shima H, Suga S, Ogino M, Shimizu K, Toya S, Nagao M, Kawase T: Transient forebrain ischemia induces expression of serine/threonine protein phosphatase 1 mRNA in the vulnerable regions of gerbil brain. *Neurosci Lett* 2002; 325:115-8

31. Sato Y, Laskowitz DT, Bennett ER, Newman MF, Warner DS, Grocott HP: Differential cerebral gene expression during cardiopulmonary bypass in the rat: Evidence for apoptosis? *Anesth Analg* 2002; 94:1389-94

32. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA: Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A* 1995; 92:7162-6

33. Messina S, Onofri F, Bongiorno-Borbone L, Giovedi S, Valtorta F, Girault JA, Benfenati F: Specific interactions of neuronal focal adhesion kinase isoforms with Src kinases and amphiphysin. *J Neurochem* 2003; 84:253-65

34. Patel P: No magic bullet: the ephemeral nature of anesthetic-mediated neuroprotection. *Anesthesiology* 2004; 101:1049-51

35. Angst MS, Ramaswamy B, Davies MF, Maze M: Comparative analgesic and mental effects of increasing plasma concentrations of dexmedetomidine and alfentanil in humans. *ANESTHESIOLOGY* 2004; 101:744-52

36. Ebert TJ, Hall JE, Barney JA, Uhrich TD, Colino MD: The effects of increasing plasma concentrations of dexmedetomidine in humans. *ANESTHESIOLOGY* 2000; 93:382-94