Letter to the Editor

I₁ imidazoline receptor-mediated effects on apoptotic processes in PC12 cells

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Dear Editor,

Imidazolines compounds elicit diverse effects on cell growth and death. At least, three subtypes of imidazoline receptors I₁R, I₂R and I₃R.^{1–3} Some imidazoline ligands are potent inhibitors of vascular smooth muscle cell proliferation through the activation of I₂R.² Other imidazoline compounds inhibited the growth of clonal pancreatic β -cells by an unknown mechanism.⁴ In contrast, independent of imidazoline receptors, some imidazoline compounds protect cerebellar granule cells against glutamate-induced neurotoxicity.⁵ At present, no effect dependent on I₁R stimulation on survival or cell death is described. However, a recent study has demonstrated a neuroprotective effect of several imidazoline, having affinity for I₁R, on cytotoxicity induced by naphthazarin in astrocytes.⁶ But, this neuroprotection does not depend on I₁R stimulation. Our laboratory is interested in I₁ imidazoline receptor. I₁R are involved in the central regulation of blood pressure and cardiovascular function.⁷ Some cell lines, such as PC12 cells, express the I₁R localized on plasma membranes.¹ In these cells, several transduction pathways associated with the receptors have been described: imidazolines, such as benazoline, increase the diacylglycerol level in the cells through the activation of a phosphatidylcholine-specific phospholipase C (PC-PLC) and decrease concentration-dependently the forskolin-stimulated cAMP level in insensitive-pertussis toxin manner.¹ These two transduction pathways are largely involved in the signalling of cell growth, cell transformation and cell death. The present work addressed the issue of the implication of I₁R in death or growth of PC12 cells.

To examine a potential role for I₁R in protection against apoptosis, we used cell death model induced by serum deprivation. When PC12 cells are deprived of serum, they undergo apoptosis or programmed cell death.⁸ The death was assessed by counting trypan blue excluding cells with a hemacytometer. The apoptotic population was quantitatively evaluated by Hoechst staining nuclei. Under serum-deprivation condition, we observed a significant decrease of viable cells time-dependently as compared with control time 0 (Figure 1a, left panel). From 8 h, the cell death was yet 40% and reached 65% at 48 h. This cell death was associated with an enhancement of chromatin condensation ($9 \pm 2.8\%$) as compared with serum-containing condition ($0.5 \pm 0\%$) (Figure 1a, middle panel). Apoptosis process induced by serum deprivation is known to be mediated by the mitochondrial pathway in which caspases 3 and 9 were activated.⁸ So,

an increase of caspase-3-like activity was considered as a second index of apoptosis. We therefore measured the activity of this enzyme after serum deprivation and compared to serum-containing condition. From 3 h, caspase-3-like activity increased significantly in serum-deprivation condition to reach a plateau at 6 h and remained until 24 h in comparison with serum-containing condition (Figure 1a, right panel). Then, these results showed that PC12 cells died by apoptosis under serum-deprivation condition.

To examine I₁R implication on PC12 cell death, we used benazoline, an agonist of two transduction pathways, which has a high affinity and is selective for I₁R.¹ Under normal growing conditions, that is in the presence of serum, benazoline did not have proapoptotic effect on PC12 cells (data not shown). Under apoptosis condition, benazoline (10^{-6} M) was added at the onset of serum deprivation and led incubate until 48 h. Benazoline enhanced significantly cell death induced by serum deprivation at 8 and 24 h (Figure 1b, upper panel). After 24 h, this effect disappeared and returned to the level of serum-deprivation condition. The increase was 18 and 25% at 8 and 24 h, respectively, as compared with control in the absence of serum for the same time. This enhancement of cell death by benazoline was associated with an increase of chromatin condensation ($26 \pm 2.6\%$) at 24 h (Figure 1b, lower panel). Since caspase-3 was activated in this apoptotic process, we next measured its activity. The cells were incubated 6 h because we had the maximal effect of serum deprivation (Figure 1a). After 6-h serum-deprivation in the presence of benazoline, caspase-3 like activity was significantly enhanced concentration-dependently in comparison with serum-free control with an EC₅₀ of $5.75 \pm 2.99 \times 10^{-11}$ M and a maximal of $103 \pm 19\%$ (Figure 1c, upper panel). These results proved that benazoline not only enhanced PC12 cells apoptosis associated with caspase-3 activation, but also accelerated the process in the first 24 h.

In order to prove that the effects of benazoline were due to I₁R stimulation, different experiments were performed in the presence of efaroxan, which is an I₁R reference and selective antagonist.⁹ Under normal growing conditions, efaroxan (10^{-5} M) had no pro-apoptotic effect on PC12 cells (data not shown). This result corroborates previous data obtained with efaroxan.⁴ Under serum-free conditions, efaroxan had no effect by itself but blocked both effects of benazoline: the enhancement of cell death and the increase of caspase-3 like activity irrespective of the benazoline concentration

(Figure 1c). As efaroxan, which was shown to antagonize the PC-PLC pathway associated with I_1R , blocked benzazoline effects, we hypothesized that the apoptotic pathway associated with I_1R may involve PC-PLC.⁹ To check this

hypothesis, we used D609 that is a selective inhibitor of PC-PLC.⁹ In apoptotic conditions, D609 had no effect by itself as compared with serum-deprivation condition (Figure 1d). This inhibitor prevented completely the increase of caspase-3 like

a

b

c

d

e

activity induced by benazoline (Figure 1d). Taken together, these results showed that activation of I₁R facilitates apoptosis under serum-deprivation condition and that this effect involves the PC-PLC pathway.

There are two major signalling pathways of apoptosis: (i) the mitochondrial pathway activated by serum deprivation; (ii) the death receptor pathway activated by cytokines such as tumor necrosis factor α (TNF α). These pathways converge to caspase-3 that then induces downstream events involved in a common apoptotic process. It has previously been shown that PC12 cells treated with TNF α underwent apoptosis.¹⁰ In the present study, TNF α (50 ng/ml) increased significantly the caspase-3-like activity in comparison with control conditions from the 24th h onwards (Figure 1e). This increase of caspase-3-like activity induced by TNF α was prevented by benazoline (10^{-6} M), which was added at the onset of incubation (Figure 1e). In order to prove the I₁R implication in the protective effect of benazoline, we used the selective antagonist efaroxan. It had no effect by itself but blocked the decrease of caspase-3-like activity induced by benazoline (Figure 1e). We confirmed the protective effect of benazoline on cell viability by counting trypan blue excluding cells. In the presence of TNF α , we observed a significant time-dependent decrease of the number of viable PC12 cells. When adding TNF α to the serum, it varied from 101.2 ± 3.09 to $83.48 \pm 2.37\%$ (17% of decrease, $P < 0.01$, $n = 3$ in duplicate) at 24 h and from 135 ± 6.8 to $107.6 \pm 3.1\%$ (20% of decrease, $P < 0.01$, $n = 3$ in duplicate) at 48 h. This decrease of the number of viable cells was prevented by benazoline (10^{-6} M); a complete prevention was even observed at 48 h. These results indicated that benazoline was able to protect PC12 cells against TNF α cell death.

Finally, benazoline had two opposite effects mediated by I₁R stimulation: induced a facilitation of serum-deprivation-induced apoptosis and a protective effect against cell death induced by TNF α . Similar effects were already described in transformed keratinocytes cell line.¹¹ In fact, interleukin-1 protected these cells from Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) and CD95-induced apoptosis. In contrast, it enhanced keratinocyte apoptosis induced by ultraviolet radiation.¹¹ Under serum-deprivation conditions, BH₄ (tetrahydrobiopterin) had an effect similar to that of benazoline in PC12 cells. In fact, this compound also enhanced the rate of death in PC12 cells but only in apoptotic conditions associated with serum deprivation. The enhancement of cell death was completely abolished by a caspase inhibitor. BH₄ was unable to induce apoptosis in the presence of growth factors.¹² The effects of benazoline on apoptosis induced by TNF α were similar to the protective effects of some imidazoline-like compounds against cytotoxicity induced by naphthazarin in astrocytes.⁶

In summary, we have shown for the first time that I₁R might be involved in some apoptotic processes. The I₁ effects of imidazoline drugs on apoptotic processes clearly depend on the cell death model.

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Figure 1 (a) Model of apoptosis induced by serum deprivation. PC12 cells were treated either with serum-free medium or with serum-containing medium for different times. *Left panel*: Survival curves of cells. Cells were harvested after 0, 8, 24, 30 and 48 h of culture. Viable cells excluding trypan blue were counted with a hemacytometer. The results are expressed as percentage of control at time 0 for each condition. Values are the mean $\pm$ S.E.M. of four experiments, each one performed in duplicate. *, #: $P < 0.01$; *, as compared with control time 0; #, as compared with serum-containing condition for the same time. *Middle panel*: Hoechst staining nuclei. Cells were treated for 24 h. Then, cells were stained with Hoechst 33342. The results are expressed as percentage of fragmented nuclei evaluated under each condition, by counting cells at least 10 randomly chosen fields. Values are the mean $\pm$ S.E.M. of three experiments, each one performed in duplicate. *Inset*: a representative Hoechst staining of PC12 cells treated with serum-free medium among three is shown. Arrows show condensed and fragmented nuclei. *Right panel*: Measure of caspase-3-like activity. PC12 cells were incubated for 0, 3, 6 and 24 h. For each time, caspase-3-like activity was measured by the Z-DEVD-pNA (20 μ M) cleavage in cell extracts. This activity was calculated after subtraction of the absorbance (OD) obtained in the presence of caspase-3 enzyme inhibitor Ac-DEVD-CHO (10 μ M) measured in parallel for each sample: OD₄₀₅ in the absence of inhibitor – OD₄₀₅ in the presence of inhibitor per μ g protein performed with Bradford method for each sample. The results are expressed in arbitrary units. Values are the mean $\pm$ S.E.M. of three experiments, each one performed in triplicate. *, #: $P < 0.05$; *, as compared with control time 0; #, as compared with serum-containing condition for the same time. w/o serum: without serum. (b) Benazoline enhanced apoptosis under serum-free condition. Cells were incubated with serum-free medium either in the absence or in the presence of benazoline (10^{-6} M). *Upper panel*: Viable cells were counted as described in (a). The results are expressed as percentage of control at time 0 for each condition. Values are the mean $\pm$ S.E.M. of five experiments, each one performed in duplicate. *, $P < 0.01$, as compared with serum-free condition for the same time. *Lower panel*: Cells were stained with Hoechst 33342 after 24-h incubation. The results are expressed as percentage of fragmented nuclei as described in (a). Values are the mean $\pm$ S.E.M. of three experiments, each one performed in duplicate. *Inset*: a representative Hoechst staining of PC12 cells treated by benazoline. Arrows show condensed and fragmented nuclei. w/o serum: without serum. (c) Efaroxan blocked the enhancement of apoptosis cell death induced by benazoline. Cells were treated with benazoline either in the absence or in the presence of efaroxan (10^{-5} M). *Upper panel*: Caspase-3-like activity was measured as described in (a) after 6-h incubation. The results are expressed as percentage of control values obtained in serum-free condition at 6 h. Values are the mean $\pm$ S.E.M. of 4–5 experiments, each one performed in triplicate. *Lower panel*: Viable cells were counted as described in (a) after 24-h incubation. The results are expressed as percentage of control at time 0 for each condition. Values are the mean $\pm$ S.E.M. of four experiments, each one performed in duplicate. *, $P < 0.05$, as compared with serum-free condition; **, $P < 0.01$, as compared with benazoline condition. w/o serum: without serum; w/o benazoline: without benazoline. (d) PC-PLC inhibitor D609 blocked benazoline effect on caspase-3-like activity. PC12 cells were preincubated with D609 (10 μ M) for 30 min. Then, cells were treated with or without benazoline (10^{-6} M) either in the absence or in the presence of D609 (10 μ M) for 6 h. Caspase-3-like activity was measured as described in (a). The results are expressed as percentage of control values obtained in serum-free condition at 6 h. Values are the mean $\pm$ S.E.M. of three experiments, each one performed in triplicate. **, $P < 0.01$, as compared with benazoline condition; NS: no significant. w/o serum: without serum; w/o benazoline: without benazoline. (e) Benazoline prevented the increase of caspase-3-like activity induced by TNF α : this effect was blocked by efaroxan. PC12 cells were treated with TNF α (50 ng/ml) in serum-containing medium (2.5%) in the presence of benazoline (10^{-6} M), of efaroxan (10^{-5} M) or of both for 24 h. Caspase-3-like activity was measured as described in (a). The results are expressed in arbitrary units. Values are the mean $\pm$ S.E.M. of four experiments, each one performed in triplicate. *, $P < 0.01$; #, $P < 0.02$; NS: no significant.

*L Dupuy¹, D Urosevic¹, H Greney¹, W Quaglia²,
M PIGIN², L Brasil³, M Dontenwill⁴ and P Bousquet^{*,1}*

¹ Laboratoire de Neurobiologie et Pharmacologie Cardiovasculaire, INSERM E-0333, Université Louis Pasteur, Faculté de Médecine, 11 rue Humann, 67000 Strasbourg, France

² Dipartimento di Scienze Chimiche, Università di Camerino, 62032 Camerino, Italy

³ Dipartimento di Scienze Farmaceutiche, Università di Modena, 41100 Modena, Italy

⁴ Present address: Laboratoire de Pharmacologie et Physicochimie des Interactions Cellulaires et Moléculaires, UMR 7034, Faculté de Pharmacie, 67400 Illkirch, France.

* Corresponding author: P Bousquet, Laboratoire de Neurobiologie et Pharmacologie Cardiovasculaire, INSERM E-0333, Faculté de Médecine,

11 rue Humann, 67000 Strasbourg, France. Tel: + 33 3 90243390;
Fax: + 33 3 90243388; E-mail: Pascal.Bousquet@medecine.u-strasbg.fr

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