Letter to the Editor

Atrial natriuretic peptide induces cell death in human hepatoblastoma (HepG2) through the involvement of NADPH oxidase

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Dear Editor,

Atrial natriuretic peptide (ANP), a hormone mainly produced by cardiomyocytes and interacting with specific receptors located within the plasma membrane of target cells, plays an important role in several renal and cardiovascular mechanisms.¹ Two major subtypes of ANP receptors have been characterized: NPR-A receptors, which activate guanylate cyclase (GC), giving rise to cyclic GMP after binding ANP at pharmacological concentrations (10^{-6} – 10^{-8} M), and NPR-C receptors, which modulate intracellular cAMP or membrane lipid turnover through the activation of specific phospholipases^{1,2} after binding the hormone at physiological concentrations (10^{-9} – 10^{-11} M). The presence of ANP receptors in different organs and tissues, including lung and liver,¹ has been related to the ANP ability to negatively regulate cell growth by different pathways.³ Among others also the membrane-bound NADPH oxidase plays an important role in cell growth regulation through the production of reactive oxygen species (ROS). The enzyme activation proceeds at the plasma membrane level through a multistep assembly of several cytosolic factors and small GTP-binding proteins, such as Rap1A and Rac.⁴ In particular, in HepG2 cells, NADPH oxidase Rac1 subunit is involved in the generation of ROS, which, when in excess, can switch apoptosis to necrosis by the inhibition of caspase-3.⁵ To identify a possible link between ANP, ROS production and cell growth, we analyzed in more detail the role of ANP in cell death mechanisms and the possible relationship between the physiological concentration (10^{-10} M) of ANP and ROS production in HepG2 cells.

Cell treatment with ANP 10^{-10} M for 6 h increased ROS production. Such an increase was totally abolished after cell pretreatment with 10^{-7} M DPI or 10^{-6} M AEBF, two NADPH oxidase inhibitors, or 100 ng/ml *Clostridium difficile* toxin A, an inhibitor of NADPH oxidase Rac subunit, and only reduced after 500 U/ml catalase pretreatment, a scavenger of hydrogen peroxide (Figure 1a, left panel). The same treatments were carried out to define ANP effects on HepG2 cell proliferation. The catalase pretreatment only partially inhibited ANP effects on cell growth, while DPI or AEBF as well as *C. difficile* toxin A pretreatment totally abrogated hormone effects (Figure 1a, right panel). ANP effects were mimicked by 10^{-10} M cANF, an ANP analogue with high specificity for NPR-C receptor involved in the hormone physiological

effects, while no effects were detectable in the presence of 50 μ M 8-Br-cGMP, a nonhydrolyzed analog of cGMP involved in ANP pharmacological effects (data not shown). To clarify whether other than NADPH oxidase sites might play a role in ANP-induced ROS production, we investigated hormone effects in the presence of inhibitors of either mitochondrial or nonmitochondrial ROS-generating enzymes (see Table 1 of Supplementary Information). To definitively demonstrate the ability of ANP to activate NADPH oxidase, we analyzed the enzyme activity *in vitro* (Figure 1a, under panel). The possible ANP effects on Rac1 expression and protein levels, using RT-PCR and Western blot analysis, were assessed. As shown in Figure 1b, upper panel, Rac1 mRNA was overexpressed in HepG2 cells after ANP and cANF treatment, while no effects were observed in the presence of 8-Br-cGMP. A significant increase was also observed in Rac1 protein levels after ANP treatment with respect to untreated cells (Figure 1b, under panel). To test whether ANP-induced cell growth inhibition could be associated to apoptosis, DNA fragmentation and TUNEL labelling were assessed. Results, reported in Figure 1c, showed no significant differences in DNA fragmentation with respect to untreated cells after ANP treatment (lane 3 *versus* lane 2) (left panel), while only a small percentage of cells (about 1%) exhibited TUNEL-positive signals in ANP-treated cells (right panel). Interestingly, pretreatment with catalase significantly increased the number of TUNEL-positive cells ($\sim 10\%$) (right panel). Since excessive ROS production may interfere with apoptotic death program by inhibiting caspase-3,⁵ ROS levels were lowered by catalase to evaluate ANP effects on caspase-3 expression. A significant activation of caspase-3 was detectable only when cells were pretreated with catalase and then stimulated with ANP (Figure 1d, upper panel). This was consistent with results obtained by monitoring ANP effects on caspase-3 activity, as evaluated by the increase in fluorescence generated by fluorogenic specific substrate Ax-DEVD-AFC cleavage (Figure 1d, under panel). Finally, ANP effects on lactic dehydrogenase (LDH) release in the presence or absence of catalase and superoxide dismutase (SOD), a scavenger of superoxide anion, were evaluated to test whether the ANP-induced cell growth inhibition could be associated to necrosis. An ANP-induced significant increase, reaching a maximum at 6 h in the outside enzyme activity, was

detected (Figure 1e, inset). Cell pretreatment with catalase or SOD significantly decreased ANP-induced LDH release (Figure 1e), which was totally abrogated in the presence of catalase plus SOD.

These results suggest that ANP, increasing ROS levels through NADPH oxidase activation, is able to induce cell death by necrosis in cells phenotypically modified, such as HepG2 cells. This effect is not in contrast with data showing an ANP cytoprotective action in liver cells subjected to ischemia-reperfusion injury,⁶ because the spectrum of biological actions of ANP appears to follow different pathways depending on cell phenotype.^{7,8} For example, cells which are known to undergo phenotypic modulation, such as those in 'in vivo' atheromatous lesions and cancerous cells such as HepG2, are highly sensitive to ANP,^{8,9} because they express a high number of NPR-C receptors. Therefore, it is reasonable to hypothesize that the different outcome in viability of hepatoblastoma cells and hepatocytes could be determined by the presence of a threshold signal, dependent on the higher expression of NPR-C receptors and increased intracellular ROS levels in HepG2 cells. Here, we show for the first time that ANP induces cell death via NADPH oxidase activation. The ability of ANP to modulate cell growth through high levels of ROS is, very likely, needed to keep caspase-3 enzyme

inactivated and to switch cell death from apoptosis to necrosis. This observation may be relevant in different pathological conditions, such as atherosclerosis and tumors, in which a strong resistance to apoptosis is present and necrotic death represents the only pathway to eliminate pathological cells.

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Figure 1

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>).

Figure 1 (a) ANP effects on ROS production and HepG2 cell growth. Upper panel: HepG2 cells (1×10^6) were initially grown in RPMI-1640 plus 10% FCS and then starved for 24 h to promote their synchronization and to rule out possible interferences with cell growth. Cells were labelled with DCF-DA ($10 \mu\text{M}$ for 60 min at 37°C) in the dark to evaluate ROS production (left panel) or with [^3H]thymidine ($1 \mu\text{Ci/ml}$) to evaluate cell growth (right panel). Cells were challenged with 10^{-10} M ANP for 6 h. When requested, cells were pretreated with 500 U/ml catalase or 10^{-7} M DPI or 10^{-6} M AEBSF for 1 h, or 100 ng/ml *C. difficile* toxin A for 24 h. For ROS generation, data represent the fluorescence intensity (expressed as fluorescence units (FU)) of cells loaded with DCF-DA only (Control), or also treated with $300 \mu\text{M}$ H_2O_2 for 1 h as positive control. Data are the mean $\pm$ S.D. of four different experiments carried out in duplicate. $^*P < 0.05$, as from data analysis with respect to untreated cells (Control). Under panel: ANP effects of ANP on NADH/NADPH oxidase activity. HepG2 cells were challenged or not (Control) with 10^{-10} M ANP for 6 h before homogenization. Superoxide production was measured in the presence of either $100 \mu\text{M}$ NADPH or $100 \mu\text{M}$ NADH through a luminescence assay using lucigenin. The amount of superoxide anion produced was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase as described by Ohara *et al.*¹⁰ (b) Rac1 expression and activity after ANP treatment. Upper panel: Total RNA was extracted by the Proteinase K-SDS method¹¹ from HepG2 cells (about 1×10^7 cells) treated with 10^{-10} M ANP or cANP or $50 \mu\text{M}$ 8-Br-GMPc for 6 h or untreated (C). The RNA ($2 \mu\text{g}$) was DNase treated (Amersham Pharmacia Biotech), reverse-transcribed into cDNA and PCR-amplified as described previously.¹² Specific primers designed to amplify the Rac1 mRNA or the ribosomal protein S6 mRNA were used. The expression levels of Rac1 mRNA in HepG2 cells were quantified by normalizing its respective cDNA level to the housekeeping human rpS6 cDNA level. The amplification cycle numbers were preliminarily optimized for each PCR reaction, so as to avoid saturation (30 cycles for Rac1 and 20 for S6). Amplified fragments were separated on 5% polyacrylamide gel and quantified by a Phosphor-Imager. Values represent the mean $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$, as from data analysis with respect to untreated cells (c). Under panel: For Rac1 immunoblot, HepG2 cells were grown to 80% confluence and made quiescent as reported above before stimulation with 10^{-10} M ANP for 15 or 30 min. Cells were incubated overnight at 4°C with $25 \mu\text{l}$ of agarose conjugated with anti-Rac1 antibody (Santa Cruz Biotechnology, CA, USA) and then washed with lysis buffer containing 0.05% SDS, and twice with Tris-HCl 10 mM, pH 7.5. The agarose beads were then resuspended in $20 \mu\text{l}$ of $1 \times$ SDS sample buffer, boiled for 5 min, loaded on 15% SDS-PAGE gel and proteins were transferred to nitrocellulose. After blocking for 1 h in PBS containing 5% dry milk and 0.1% Tween20, the membrane was incubated with anti-Rac1 antibody (1:500) overnight. Rac1 protein was detected by enhanced chemiluminescence. Each experiment was repeated at least three times. (c) Absence of DNA fragmentation and apoptosis after ANP treatment. Left panel: DNA ($10 \mu\text{g}$) extracted from HepG2 cells (2×10^7) treated with 10^{-10} M ANP for 6 h (lane 3) or untreated (lane 2) were resolved by electrophoresis on 1.8% agarose gel and visualized with ethidium bromide. In lane 1, DNA molecular markers were loaded (100 bp DNA ladder). Right panel: Apoptosis in individual cells was assessed by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) technique, as described by Gavrieli *et al.*¹³ HepG2 cells were untreated (Control) or stimulated with 10^{-10} M ANP for 6 h (ANP) after incubation with 500 U/ml catalase (ANP + catalase). Results were evaluated by counting positively stained nuclei per microscopic field. (d) ANP effects on caspase-3 activation in HepG2 cells. Upper panel: HepG2 cells pretreated with 500 U/ml catalase for 1 h were challenged with 10^{-10} M ANP for 6 h and analyzed by Western blot. In all, 30–50 μg of whole-cell lysate was subjected to 10% acrylamide SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-caspase-3 antibody (1:2000) overnight at 4°C . A horseradish peroxidase-conjugated secondary antibody (1:2500) was applied for 2 h at room temperature, and the membranes were analyzed by ECL. The asterisk points to the caspase-active fragment. Each experiment was repeated at least three times. Under panel: The caspase-3 activity was measured fluorometrically in cell extracts by assaying the cleavage of the fluorogenic specific substrate Ac-DEVD-AFC, by a Perkin-Elmer luminescence using excitation wavelength 440 nm and emission wavelength 500 nm. $^*P < 0.05$, as from data analysis with respect to untreated cells (c). (e) ANP effects on lactic dehydrogenase release. LDH activity was determined in both culture medium and intracellularly. HepG2 cells, pretreated or not with 500 U/ml catalase or 10^{-6} M SOD for 1 h and challenged with 10^{-10} M ANP for 6 h, were gently scraped in the culture medium and an aliquot of the suspension was utilized for total enzyme assay after cell lysis. Another aliquot was centrifuged at $700 \times g$ for 10 min. The supernatant was then used for extracellular enzyme assay. The extracellular enzyme activity was expressed as percentage of the total activity. Inset: A confluent monolayer of HepG2 cells was treated for different experimental times (1–9 h) with 10^{-10} M ANP pretreated or not with 500 U/ml catalase for 1 h. Data represent one out of, at least, three different experiments. $^*P < 0.05$, as from data analysis with respect to time 0 (Control)