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Short communication

Effects of opioids on proximal renal tubular cells undergoing ATP depletion

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ABSTRACT

This study investigated the effect of morphine, fentanyl, butorphanol and buprenorphine on viability and caspase-3 activity in renal proximal tubular cells exposed to opioids for 2 h before or 12 h after chemical anoxia. Cell viability decreased regardless the treatment although intracellular ATP content was elevated in morphine and fentanyl pre-treated cells at 12 h. Anoxia increased caspase activity but this effect was significantly reduced in cells treated before or after with morphine, fentanyl and in cell treated with butorphanol for 12 h. No influence of buprenorphine was detected. Morphine, fentanyl and butorphanol might have protective effects during kidney ischemia.

Ischemia and reperfusion (IR) injury is unavoidable event during solid organ transplantation and it may affect graft function. Caspases are the main effectors of apoptosis and their activity increases after intracellular ATP depletion due to IR injury (1–4).

In vivo, κ opioid receptors (κORs) have been identified in different organs including heart and kidney (5) and the use of selective agonists of these receptors showed protection against IR damages. Inhibition of caspases and attenuation of cardiomyocytes apoptosis were observed when κOR agonists were administered before or immediately after left anterior descending coronary artery legation in rats (3,4). Morphine which shows high affinity for κOR (6,7), administered before renal artery occlusion improved organ function and attenuated histological lesion score compared to untreated control group (8).

The aim of this in vitro study was to investigate the effects on cell viability and caspase activation of different opioids administered before or after chemical anoxia induced by ATP depletion in proximal tubular kidney cells.

Opossum kidney proximal tubular (OK) cells, which naturally expresses functioning κOR (7), were purchased from the American Type Culture Collection (Manassas, VA, USA) and seeded in 96-well plates at a concentration of 7×10^3 cells/well. Cells were incubated until confluence in DMEM/high glucose (Euroclone, Milan, Italy) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Euroclone, Milan, Italy) at 37 °C in 5% CO_2/95% air.

Two different experimental conditions were tested. In the first case (PRE), cells were exposed for 2 h to a culture medium containing morphine, fentanyl, butorphanol or buprenorphine at 10^{-8} M. Chemical anoxia was induced by 2 h-exposure to antimycin A (10 μM) and 2-deoxy-D-glucose (2 mM) (Sigma–Aldrich, St. Louis, MO, USA) in PBS to simulate ischemia as previously reported (1). Cells were then cultured for additional 12 h in freshly made medium.

In the second experimental condition (POST), after 2 h of ATP depletion, cells were exposed to medium containing opioids at 10^{-8} M for the following 12 h. For each plate, wells containing cells not exposed to chemical anoxia and cells undergoing only ATP depletion were used as negative and anoxic control, respectively.

In each experimental condition, ATP, cell viability and caspase assay were performed immediately and 12 h after the simulated ischemia.

ATP cell content was measured by luciferase assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega Corporation, Madison, WI, USA) according to manufacturer's guidelines. OK cells were seeded in opaque-walled plates and ATP content was evaluated by recording luminescence in each well with a multiplate luminometer (Victor^{3}TM, PerkinElmer Life Sciences, Monza, Italy).

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Viability and caspase activity in OK cells were quantified with a CellTiter-Blue assay combined with Apo-ONE Homogeneous Caspase 3/7 assay (Promega Corporation Madison, WI, USA). Viability was measured by adding 20 μl of Celltiter-Blue reagent to each well and recording fluorescence (560em/590ex) after 2 h. Caspase activity was measured in the same wells adding the fluorescent substrate (Z-DEVD-rhodamine 110). Caspase activation, resulting in the release of rhodamine110 from its substrate, was measured by excitation at 485 nm (emission 527 nm) via fluorimetry (Victor^{3}TM PerkinElmer Life Sciences, Monza, Italy) and normalized for the number of viable cells, as determined by CellTiter-Blue assay.

GraphPad Prism 6 (GraphPad Software, St. Diego, CA, USA) was used for statistical analysis. One-way ANOVA followed by Dunnett's multiple comparisons was used to compare means. Data are expressed as mean ± SD (standard deviation) percentage of relative luminescence or fluorescence units normalized for the untreated control cells. Statistical significance was defined as $P < 0.05$.

Immediately after two hours of chemical anoxia, an 85% decrease in ATP content was observed regardless of treatment (Fig. 1A). Nevertheless, 12 h following anoxia, cells pre-treated with morphine and fentanyl presented a statistically significant increase in ATP content compare to the anoxic control. The use of the same opioids in the post-anoxic period did not cause any ATP improvement (Fig. 1B). At 12 h, butorphanol exposure did not improve ATP content regardless of the treatment scheduled, while ATP content decreased in cells treated with buprenorphine both before and after chemical anoxia (Fig. 1B).

Cell viability decreases by 40% compared to negative control in all treatment groups both immediately and 12 h after simulated ischemia (Fig. 1A,B). However, a slight but significant increase in cell viability, evaluated immediately after the anoxic period was recorded in morphine-pretreated cells compared to the anoxic control (Fig. 1A). This effect was not detected in the following 12 h. In addition, 12 h after chemical anoxia, a statistically significant decrease in cell viability was observed in buprenorphine post-treated cells (Fig. 1B).

Fig. 2 shows caspase-3 and -7 activity measured immediately after (Fig. 2A) or at 12 h (Fig. 2B) following ATP depletion. Morphine and fentanyl exposed cells had a lower caspase activation compared with the anoxic control at all time points considered and independently of the scheduled treatment. Butorphanol significantly inhibited the caspase activity only when added to cells for 12 h after ATP depletion (Fig. 2B). Buprenorphine did not abolish caspase activity compared with the anoxic control cells at any time point considered.

In vivo, complete obstruction of renal blood supply caused caspase-3 activation, apoptosis and impairment of kidney function in rats (2,8). Also in this in vitro study an anoxic insult caused an increase in caspase-3 activity that was attenuated if morphine or fentanyl were administered 2 h before or for 12 h after ATP depletion. This is the first report of a direct modulation of opioids on caspase activity after chemical anoxia in proximal tubular cells as it was observed in other organs (3,4).

Pre-treatment with morphine was also associated with an improvement in cell viability at the end of the anoxic event, suggesting an early protective effect of some opioids against ischemic injury (4,8).

Cell oxidative stress and reactive oxygen species (ROS) production are observed during prolonged ATP depletion or during cell re-oxygenation after ischemia; reduction in ROS generated minimize cell injuries via inhibition of mitochondrial dysfunction and caspase activation (9). Agonists of κOR administered before anoxia decrease apoptosis as observed in different tissues (2–4,10). Opioid ability to activate preventively the mechanisms that inhibit ROS production during and after the ischemic period may explain the protective effect of morphine and fentanyl. Moreover these opioids preserve mitochondrial membrane potential and maintain ATP production by enhancing mitochondrial ATP potassium channel

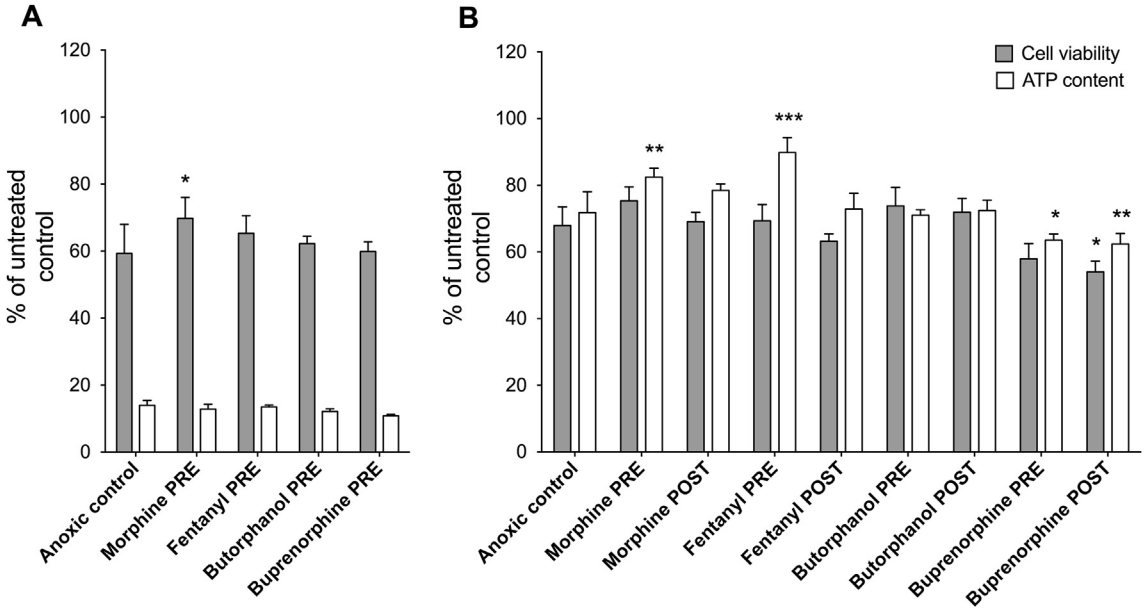

Fig. 1. Effect of opioids on viability (gray bars) and ATP content (white bars) in OK cells after 2 h of chemical anoxia. Cells underwent opioid exposure at 10^{-8} M, 2 h before (PRE) or for 12 h after (POST) 2 h of chemical anoxia. (A) Immediately after anoxia, opioids exposed cells had a similar decrease in intracellular ATP content compared to untreated cells although morphine exposure statistically improved cell viability. (B) At 12 h after chemical anoxia, cells pre-treated with morphine and fentanyl had a higher ATP content while a decrease is observed in buprenorphine exposed cells both before and after anoxia. No effect of butorphanol was detected; viability was similar in all treatments but in cells exposed 12 h to buprenorphine cell viability decreased. All data are expressed as mean ± SD in five independent experiments $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ versus anoxic control.

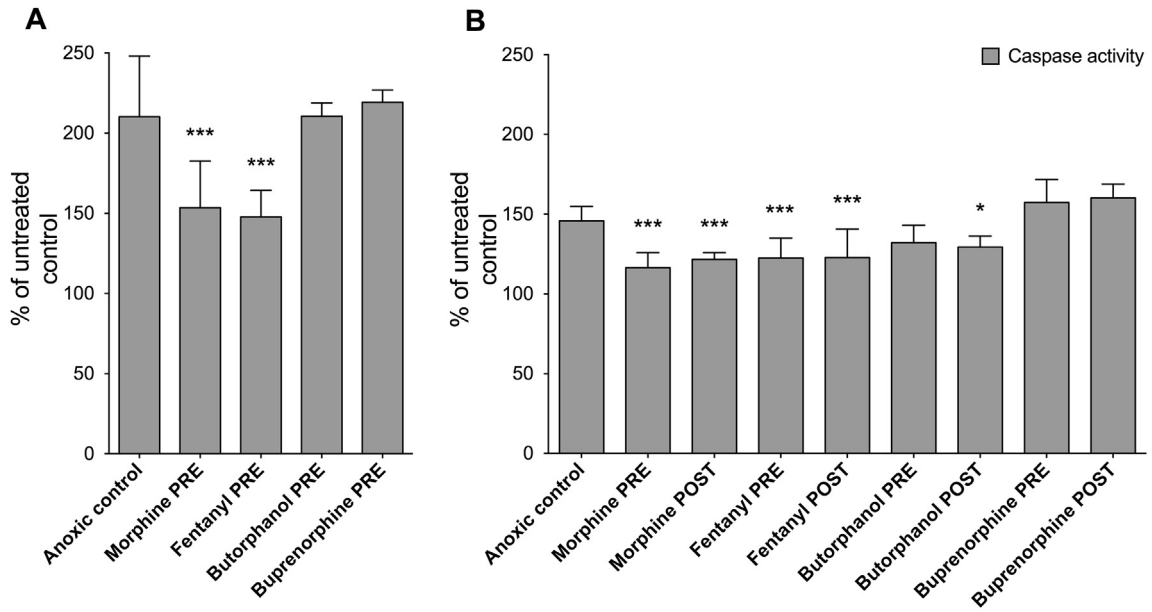

Fig. 2. Effect of opioids on caspase-3 and -7 activity normalized for viable cells following 2 h of chemical anoxia. Cells underwent opioid exposure at 10^{-8} M, 2 h before (PRE) or for 12 h after (POST) 2 h of chemical anoxia. (A) Immediately after anoxia, treatment with morphine or fentanyl but not butorphanol and buprenorphine decreased caspase activation compared to non-treated ATP-depleted cells. (B) At 12 h, cells exposed to morphine and fentanyl before or after chemical anoxia showed a decrease in caspase activity compared with non-treated ATP depleted cells; butorphanol exposure diminished caspase activity only in post-treated cells while buprenorphine treatment did not affect caspase activation. All data are expressed as mean ± SD in four or five independent experiments. $^{*}P < 0.05$ or $^{***}P < 0.001$ versus anoxic control.

opening and inhibiting mitochondrial transition pore by a protein kinase C mediated phosphorylation of GSK-3β (3,4,10,11).

Butorphanol has no effects on ATP content while buprenorphine showed a detrimental effect on kidney tubular cells viability and ATP content when added to cells for 12 h after anoxia. In a previous study buprenorphine showed a similar effect on viability and a pro-apoptotic activity on a neuronal cell line (12).

The protective effect of opioids toward IR injury was studied in vivo and in vitro in myocardiocytes (3,4,11) but few studies described the effects of these compounds against renal ischemia injury. Pazoki-Toroudi et al. (8) observed that morphine administration before 30 min of renal ischemia followed by 6 h of reperfusion attenuated histological lesion score and the increase in serum creatinine and urea. Vianna and co-workers (2) infused remifentanil before and after 45 min of renal artery occlusion in monolaterally nephrectomised rats; they observed that the percentage of early apoptotic cells were lower in animals treated with remifentanil compared with the sham group. These results agree with our observations in cells treated with morphine and fentanyl although in vivo these opioids may interact with receptor other than κOR like δ opioid receptors, that have been identified in kidney (5). Additionally in the post-ischemic period, κOR agonists may decrease ischemia-induced apoptosis by an over production of nitric oxide via a phosphoinositide 3′-kinase dependent mechanism (3).

There are no studies evaluating the effect of butorphanol on apoptosis and caspase activation after renal IR. However, κOR mediated protection was observed in rat cardiomyocytes after post-ischemic butorphanol administration (13). The intrinsic activity of butorphanol, measured by inhibition of cAMP after receptor activation, is 0.6 if considered 1 that of morphine and fentanyl (6). This weak activity might explain why no effects on caspase inhibition were observed in cells exposed for 2 h before chemical anoxia while a longer exposure may have caused the mild inhibition of caspase.

Buprenorphine is an antagonist of κOR (14) and this may explain the lack of protective effect toward caspase activity in tubular renal cells after ATP depletion. Similarly to our results, in myocardial cells under ischemic condition no decrease in caspase level was observed with norbinaltorphimine, a selective κOR antagonist (3,4). Moreover, it has been demonstrated that renal function does not improve by buprenorphine when administered after renal IR in mice (15).

In conclusion morphine and fentanyl may minimize caspase activation induced by ischemia. These opioids provided better kidney tubular cell protection when administered before ATP depletion.

Conflict of interest statement

The authors declare no conflict of interest.

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