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Gas Transmitters (CO and NO) as Factors Regulating the Skin Cells Functions

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Abstract

The paper presents the results of research conducted on cells isolated from the epidermal layer of the skin taken from the ear of domestic swine (Sus scrofa). It was checked whether the increase in the concentration of carbon monoxide (CO) in cell culture, cause changes in their viability, and will affect the changes in the production of nitric oxide. For evaluation of the viability of cells, the MTT assay was used. The concentration of a nitric oxide metabolites (NO_3/NO_2), in the cell supernatants was measured using test-Measure iT High-Sensitivity Nitrite Assay Kit.

Keywords: Cells viability, Carbon monoxide, Nitric oxide

Introduction

Carbon monoxide (CO) is a gas endogenous mediator of a variety of biological activity (Ryter, Otterbein, Morse, Choi, 2002; Wu, Wang, 2005). In biological systems it is formed as a degradation product of heme molecules in the reaction catalyzed by the heme oxygenase (HO) (Ryter et al., 2002; Bełtowski, Jamroz, Borkowska, 2004). Heme oxygenase catalyzes the reaction of degradation of heme to CO, biliverdin-IXα and ferric ions (Fe 2+). In this reaction, there are used electrons derived from NADPH - dependent reductase of cytochrome C450 (Ryter et al., 2002; Wu, Wang, 2005). Created biliverdin-IXα, is rapidly reduced to bilirubin-IXα with the participation biliverdin reductase (Bełtowski et al., 2004). This enzyme is located in the microsomal fraction of cells. It exists in three isoforms: HO-1, HO-2, HO-3, which are the products of different genes, with expression dependent on the type of cells and tissues in which they are located (Wagner et al., 2003). HO-1 is the induced form of heme oxygenase, which activation occurs by stress factors, such as inflammation, high temperature, UVB radiation, oxidative stress, increased levels of NO, as well as growth factors. CO, just like NO can activate soluble guanylyl cyclase, leading to several fold increase in intracellular cGMP. The activity of this process with partici-

pation of CO is 30–100 times lower than in the case of NO. Despite the weaker activation of guanylyl cyclase, carbon monoxide, in contrast to NO does not undergo fast inactivation, and can exert its activity for a long time (Bełtowski et al., 2004; Dulak, Józkowicz, 2003). Carbon monoxide has a number of similar properties as the nitric oxide. Both molecules participate in the regulation of the same physiological processes, they are involved, among others, in the inhibition of platelet aggregation, neurotransmission, or modulation of blood vessel function (Dulak, Józkowicz, 2003; Wu, Wang, 2005). Interaction between these two molecules is at different levels, may exhibit synergistic or antagonistic effect, thereby providing an integrated mechanism in the regulation of cellular processes (Wu, Wang, 2005). Carbon monoxide can impact directly on the production of nitric oxide (Dulak, Józkowicz, 2003). The processes of controlling of the molecules release may be associated with the induction of secretion of NO from intracellular pools or inhibition of nitric oxide synthase (NOS) (Thourp, Jones, Gross, Moore, Goligorsky, 1999). It was observed that the UVA irradiation enhanced the release of cutaneous NO stores (Juzeniene et al., 2011; Liu et al., 2014). Exposure to UVB radiation causes the release of carbon monoxide from hemoglobin (Stec, Heather, Vera, 2008). If, therefore, the nitric oxide in response to carbon monoxide is released from inside the cells, the effect of CO is similar to the effects induced by UV radiation.

The aim of the carried out experiment was to introduce the students of Biotechnology of the University of Rzeszów in the methodology of working with primary cultures and assessment of the impact of external factors on cell cultures.

The detailed aim of the study was to investigate whether the increase in the concentration of carbon monoxide in the cell culture cause changes in cells viability and affects changes in nitric oxide production.

Materials and Methods

Cell culture

The material used in the study were cells isolated from layer of the epidermis of the skin after slaughtering, collected from the ear of domestic swine (Sus scrofa). Isolation of epidermal cells was performed using the method described by Hsu et al (Hsu, Li, Herlyn, 2005). The cell culture was carried out in MEM medium Eagle's A (Sigma) supplemented with 10% FBS, 1 mM sodium puryvate (SIGMA), 1% non-essential amino acids (Sigma) and antibiotics (penicillin and streptomycin) (GIBCO). Cultures were grown in sterile culture bottles with an area of 25 cm^2 at 37°C in a humidified atmosphere containing 5% CO_2 in air.

The MTT assay

For evaluation of the viability of cells cultured *in vitro*, MTT assay was performed. The test was done after the first passage after the start of the culture. Cells were seeded in 96-well plate, 4 x 10^3 cells per well, in 100 µl of culture

medium. After 24 hours of incubation, the medium was removed and there was added a test medium containing CORM-2 (Sigma-Aldrich) at a concentrations of 25 μM, 50 μM, 100 μM, 200 μM, 400 μM, 800 μM and 1 mM. Control cells were incubated in a complete culture medium. After 24 hours of incubation, 10 μL, 12mM MTT reagent was added (Invitrogen). Cells were incubated with MTT for four hours at. 37°C. After that time the medium was removed and formazan solvent was added – 50 ml of DMSO. The absorbance was measured at a wavelength of 540 nm and a reference wavelength of 640 nm using a spectrophotometer Infinite M200 – TECAN.

Determination of the concentration of nitric oxide metabolites

The concentration of NO_3 and NO_2, a nitric oxide metabolites in the cell supernatants was measured using test-Measure iT High-Sensitivity Nitrite Assay Kit (Invitrogen). Cells were seeded in 96-well plate of 7.5 x 10^2 per well, in a volume of 100 μl of culture medium. After 24 hours of incubation, there was added the medium containing CORM-2 (SIGMA) at a concentration of 25 μM, 50 μM, 100 μM, 200 μM, 400 μM, 800 μM and 1 mM. Control cells were incubated in a complete culture medium. The test was performed at 24 and 72 hours of incubation with the tested factor. The fluorescence value was determined at a wavelength of 365/450 nm using a spectrofluorimeter Infinite M200 – TECAN. The concentration of nitrite was determined on the basis of a standard curve made with three replicates on the basis of patterns provided by the manufacturer.

Results

Cell proliferation assay

After 24 hours of incubation of the cells with CORM-2 there was observed a statistically significant increase in the absorption equivalent to growth in cell viability relative to controls at concentrations of 25 μM, 50 μM, 100 μM CORM-2, and a significant decrease in the absorption of cells incubated in the 400–1000 μM concentrations of CORM-2 (Fig. 1).

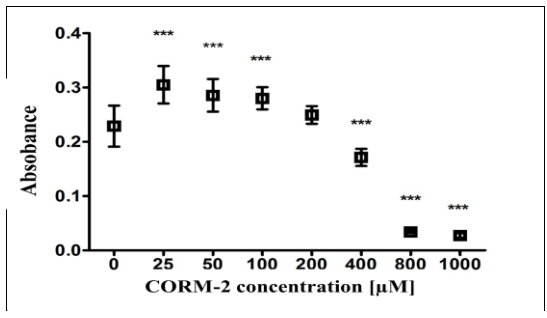

Fig. 1. MTT assay. A plot of absorbance to concentration CORM-2 used in cell culture of epidermis layer. The result are presented as mean ± SEM, *** P ≤ 0.001 vs. control

Evaluation of the impact of CO on NO secretion activity of epidermal cells

The concentration of nitrate NO_3 and NO_2, nitric oxide metabolites, was determined by using the commercial kit in cell supernatants after 24h and 72h of incubation with the tested substance. After 24 hours incubation with CORM-2 with concentrations of 200–1000 μM, the amount of nitric oxide metabolites significantly increased (Fig. 2A). After prolonged incubation of cells for 72 hours, the level of released nitrates was much higher, compared to that obtained by incubating for 24 hours. Only at the highest tested concentration of CORM-2 (1 mM) it is shown a significant decrease in nitrite and nitrate secretion by cells of the epidermal layer of the skin (Fig. 2B).

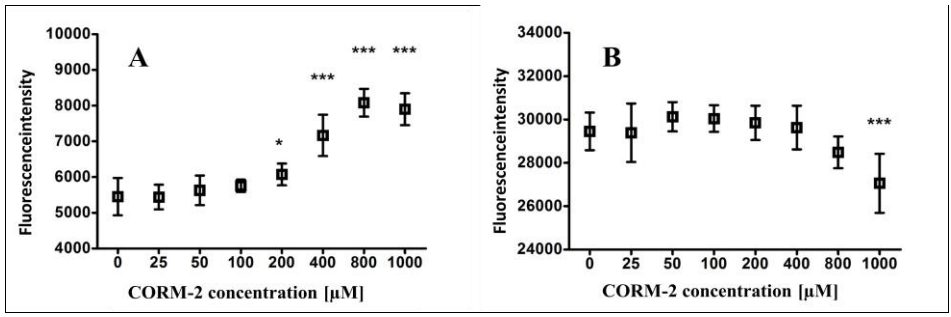

Fig. 2. The graph of the fluorescence intensity for nitrate in the cells supernatants, depending on the used concentrations of CORM-2 after 24 (A) and 72 (B) h of incubation. Results are presented as mean ± SEM, * P ≤ 0.05; *** P ≤ 0.001 vs. control

Discussion

The results obtained in this study show that 24-hour incubation of the epithelial cells CORM-2 in a concentration range from 25 μM to 100 μM, resulting in an increase in cell viability with regard to the control ($P \leq 0.001$) (Fig. 1). At concentrations of 400–1000 μM, we observed a significant decrease in cell viability ($P \leq 0.001$). Similar results were obtained in the paper of Winburg et al. (2012), working on HEK and MDCK cell lines. These results suggest that carbon monoxide in the in vitro culture of epidermal cells at concentrations up to 100 μM stimulates cell viability, whereas at concentrations exceeding 400 μM, reduces the viability or exerts a cytotoxic effect. Many research groups have confirmed the interaction between molecules of CO and NO. Among other studies carried out by Thorup et al. determine the impact of carbon monoxide at a secretory activity of endothelial cells, associated with the release of NO. They show that the CO depending on the concentration, stimulates or inhibits the release of NO. Low levels of carbon monoxide dilate supplying arterioles and releases NO from the intracellular pool. On the other hand, at higher values, CO begins to inhibit the activity of nitric oxide synthase (NOS), resulting in a reduction in the generation of NO (Thourp et al., 1999). Similar results in their study

obtained Megias et al. Incubation of Caco-2 cells with CORM-2 significantly reduced the amount of produced nitrite – secondary NO metabolites (Megias, Bussoerolles, Alcaraz, 2007). In the present study we show the operation of CORM-2 on the elution of nitric oxide by epidermal cells, determining the proportion of secondary metabolites of NO in the cell supernatants. The obtained results allow to conclude that after 24 hours of the epidermis cells incubation there is shown proportional to the quantity of CORM-2 used in experiments, increase in the concentration of NO (Fig. 2A). Statistically significant results ($P \leq 0.001$) was observed at concentrations causing cytotoxicity (200–1000 μM), which may suggest that toxic effects of CO on cells, could also be the result of raised levels of secondary metabolites of NO. We demonstrate no significant impact on the release of nitric oxide by using low concentrations of CORM-2. After 72 hours of incubation the level of released nitrates was much higher, compared to that obtained by incubating for 24 hours and only at the highest used concentration of CORM-2 (1 mM) there was shown a significant decrease of NO_3/NO_2 ($P \leq 0.001$) in relation to control (Fig. 2B). The research conducted by Romero-Graillet et al. have shown that in response to UVA and UVB human melanocytes and keratinocytes secrete nitric oxide. Released NO may play the role of mediator, increasing the synthesis of melanin by pigmented skin cells, thus demonstrating the role of autocrine and paracrine regulator of melanogenesis, induced by UV (Romero-Graillet i in., 1996). However, there are necessary more detailed studies, to be able to unequivocally state, that carbon monoxide can play a role of a molecule of a light signal in the skin.

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