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Investigation on the effects of diamide on NO production in vascular endothelial cells (VEC)

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Abstract

Nitric oxide (NO) controls several physiological functions of the cardiovascular system. The study on the effect of diamide ($N_2H_4{\cdot}H_2O$) on NO production in vascular endothelial cells (VEC) may provide significant reference for VEC's modeling in studying cardiovascular diseases. The objective of this study was to elucidate how high concentration diamide ($V_{\text{diamide}}/V_{\text{culture miedium}} = 5$ ml/l) and low concentration diamide ($V_{\text{diamide}}/V_{\text{culture miedium}} = 0.5$ ml/l) affect NO production in a human endothelial cell line (ECV304). After cells were incubated with diamide (5 or 0.5 ml/l) for 4, 6, 8 or 10 h, respectively, the amounts of NO metabolites released by the cells were quantitated and the degree of damage of VEC was observed using microscope. The results showed that NO production in VEC tended to decrease with the lapse of time in the 0.5 ml/l diamide group. In the 5 ml/l diamide group, on the contrary, NO production in VEC tended to increase with the lapse of time. At the same time, from the morphologic observation, the VEC were damaged severely after treated with 5ml/l diamide. So it could be concluded that the severe damage induced by high concentration diamide would have triggered the express of inducible nitric oxide synthases (iNOS). Just for the expresssion of iNOS, NO production in VEC treated with high concentration diamide occurred abnormally in contrast to the 0.5 ml/l group.

Keywords: Nitric oxide (NO); Nitric oxide synthases (NOS); Diamide ($N_2H_4{\cdot}H_2O$); Lipid peroxidation injury

1. Introduction

The injury and the apoptosis of vascular endothelial cells (VEC) play an important role in the occurrence and the development of cardiovascular diseases [1]. Lipid peroxidation injury is one of the key factors inducing VEC injury [2]. The rationality of VEC injury model can affect the reliability of cardiovascular drugs riddling in vitro. Diamide ($N_2H_4{\cdot}H_2O$) is a thio-oxidizing agent, which could induce lipid peroxidation injury of VEC.

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues [3,4]. The abnormality of NO production in VEC resulted from lipid peroxidation injury has close relation to cardiovascular disease [5]. NO plays an indicating role in the VEC's injury and the pathology of cardiovascular disease. At the same time, NO is the important index appraising cardiovascular drugs.

The endothelial nitric oxide synthases (eNOS) [7], which is Ca^{2+} dependent, are the master nitric oxide synthases (NOS) in VEC. NO derived from eNOS may modulate vasomotor tone and inhibit platelet, leukocyte or monocyte aggregation and adhesion to each other or the endothelium. Now inducible nitric oxide synthases (iNOS), which is not Ca^{2+} dependent [6,7], have also been found in vascular endothelium. The iNOS remained unexpressed until induced by cytokines or endotoxin (lipopolysaccharides) in some pathological process such as circulatory or inflammation. NO derived from iNOS reacts with oxygen free radical such as superoxide anion to form peroxynitrite ($ONOO^-$), which could be the main mechanism of NO toxic [8]. It was investigated respectively in this paper how high and low concentration diamide affects NO production in VEC. And combining the NO production in VEC with the morphological observation of VEC, the degree of VEC injury induced by diamide was analyzed,

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which could provide significant reference for VEC's modeling.

2. Material and method

2.1. Material

2.1.1. Cell

ECV304 of human vascular endothelial cells (HUVEC) strain purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

2.1.2. Apparatus

TC232-2E CO_2 culture tank of Sheldon Manufacturing Inc. USA; ITM-2 inverted microscope of Olympus Optical Co. Ltd., Japan.

2.1.3. Reagent

Diamide, whose molecular weight is 50.06,was purchased from Easten regent Co. Chongqing China; F-12 DMEM culture medium (catalogue no.: SH30004.01) were purchased from Hyclone Co., USA; newborn calf serum were purchased from Biotechnology Development Center, China; NO assay kit (catalogue no.: S0021) was purchased from Beyotime Biotechnology Co., Jiangsu, China.

2.2. Method

2.2.1. The culture of VEC in vitro

The VEC stored by freezing were resuscitated and put into culture flask with DMEM culture medium, which include 10% newborn calf serum, and then incubated in 5% CO_2 culture tank under the condition of 37 °C and 80% relative humidity. When cells came into logarithmic growth, they were prepared into cell suspend solution with culture medium. And then the concentration of cells was adjusted to about $1 \times 10^5\ ml^{-1}$.

2.2.2. The grouping of experiment

ECV304 were inoculated into culture flask. When cells covered the bottom of culture flask, cells were prepared into cell suspend solution which concentration was $1 \times 10^5\ ml^{-1}$. The suspend solution was inoculated into 96-well-culture plate with 0.1 ml each well. After incubated in 5% CO_2 culture tank for 24 h under the condition of 37 °C and 80% relative humidity, the upper clear liquid was thrown away. Then the experiments were carried out as the following grouping. The first one was the control group incubated without diamide. The second one was 0.5 ml/l ($V_{\text{diamide}}/V_{\text{culture miedium}} = 0.5$ ml/l) diamide injury group incubated with 0.5 ml/l diamide. The last one was 5 ml/l ($V_{\text{diamide}}/V_{\text{culture miedium}} = 5$ ml/l) diamide injury group incubated with 5 ml/l diamide. And each group had the following four subgroups: cells were incubated for 4, 6, 8 or 10 h. At the same time, each subgroup had eight parallel samples. After cells were incubated according to the grouping, NO production in VEC was detected and the cells were observed with inverted microscope by magnifying 100 times.

2.3. The measurement of NO

The transient and volatile nature of NO makes it unsuitable for most convenient detection methods, however two stable breakdown products, nitrate (NO_3^-) and nitrite (NO_2^-) can be easily detected by photometric means [6]. In this experimental, Griess method was adopted to detect NO, which is based on the chemical diazotization reaction that was originally described by Griess in 1879, which uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions [9].

After cells incubated according to the aforementioned grouping, 50 μl culture solutions of each well were collected and put into the counterpart well of another plates. Then NO production in cells was measured by Griess method and according to the indication on the NO assay kit. And the data were analyzed with statistical program for social sciences.

3. Result

3.1. The effect of diamide on NO production in VEC

As is shown in Fig. 1, the following points could be found out. In the control group, NO production in VEC was 5.76 ± 0.064 μM after VEC were incubated for 4 h, and was 6.03 ± 0.066 μM after VEC were incubated for 8 h. NO production in VEC increased slowly and slightly. In the 0.5 ml/l diamide group, NO production in VEC was 4.49 ± 0.068 μM after VEC were incubated for 4 h, which was lower than that of the control group, and was 2.23 ± 0.065 μM after VEC were treated for 10 h. With the lapse of time, the NO production in VEC tended to descend. From 4 to 10 h, NO production in VEC decreased by 50%. While in the 5 ml/l diamide group, NO production in VEC was 0.88 ± 0.063 μM after VEC were incubated for 4 h, which was far lower than that of the control group, and was 3.87 ± 0.066 μM after VEC were treated for 10 h. With the lapse of time, NO production in VEC tended to increase. From 4 to 10 h, NO production in VEC increased by 4.4 times.

3.2. The morphological observation of VEC

In the control group (see. Fig. 2), the cells appeared flat and spindly, multangular or elliptic, and arrayed inlaidly and tightly. Nucleoli located in the center of cell. The surface of cell swelled slightly and the boundaries of cells were clear. And there was no overlap growth. In the diamide 5 ml/l group (see. Fig. 3), after VEC were incubated with diamide for 10 h, the most cells shrinked and rounded. The clearance

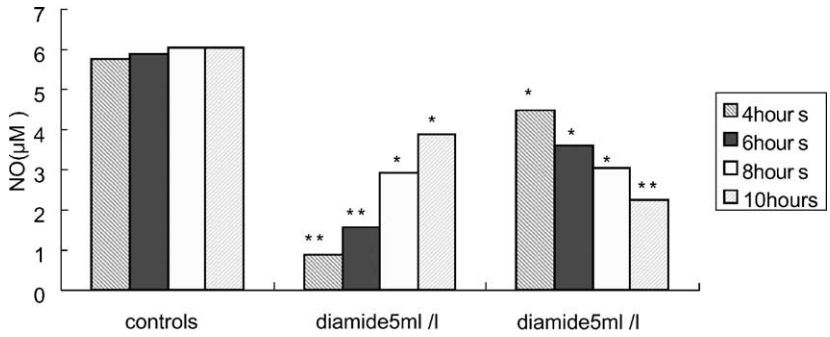

Fig. 1. VEC were treated with different time, effect of diamide on NO production in VEC. Each value was represented mean ± S.D. for eight samples. Statistical analysis compared with control group by *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

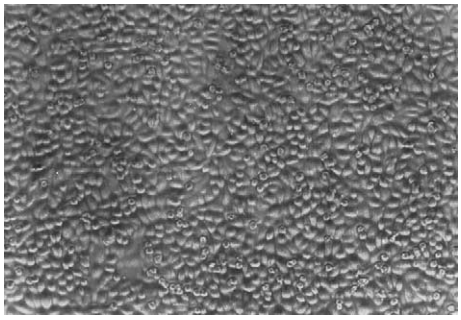

Fig. 2. VEC of control group, magnified 100 times.

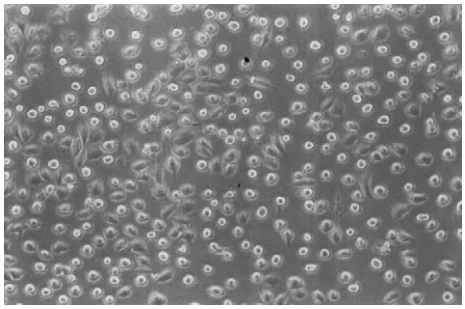

Fig. 3. VEC treatment by 5 ml/l diamide for 10 h, magnified 100 times.

of cells became larger and the figure of some cells became dark. There were dark grains in cytoplasm of some cells. In the diamide 0.5 ml/l group (see. Fig. 4), after VEC were incubated with diamide for 10 h, most cells became stellar or elliptic and only a few cells shrinked, rounded and separated. And only a few cells' clearance became larger.

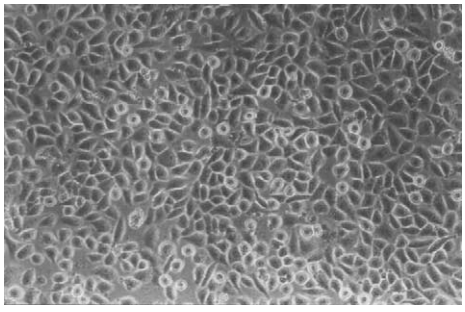

Fig. 4. VEC treatment by 0.5 ml/l diamide for 10 h, magnified 100 times.

4. Discussion

VEC is monolayer cell locating under vascular endothelium and among tissues. It is the largest incretion gland [10] and plays an important role in modulating vasomotor tone, hemostasis, anti-thrombus and maintaining natural function of vascular by secreting many active substances. Oxide-LDL, radical, abnormal blood stream and inflammatory factor are the main factors that induce VEC injury [11]. And severe injury triggers excess apoptosis of VEC. Once VEC were injured, it would result in VEC dysfunction and abnormity of active substances (such as NO, endothelin ET, thromboxane A_2 TXA_2, prostacyclin PGI_2 and so on) secretion, which trigger the occurrence and the development of diversified cardiovascular diseases. Studies showed that the functional injury of VEC could induce VEC to secrete some adhesion molecules which could result in leukocyte adhering to VEC and going in clearance under VEC to form foam cells, which is the triggering things of atherosclerosis and atherosclerosis obliterans [12,13] and is also the key factor resulting in dysfunction of hemostasis and anti-thrombus [13]. Lipid peroxidation reactions are a series of radical reactions that occur at covalent bond of unsaturated fatty acid. The investigating field about lipid peroxidation injury is very wide and, at the same time, there are many methods to model lipid peroxidation injury of cells. Diamide is a triggering reagent of radical and a sulfhydryl reagent which oxidizes sulfhydryl groups to the disulfide form [14]. The reaction could break up the balance between sulfhydryl groups and disulfide. In this way, the level of glutathione (GSH) was depressed, the radical reaction was triggered and lipid peroxidation was accelerated. And all these things resulted in VEC lipid peroxidation injury. Additionally, diamide is also the protein tyrosine phosphatase inhibitor that could active the nuclear transcription factor NF-κB [15], inducing VEC to highly impress intercellular adhesion molecule-1 (ICAM-1) and accelerating leukocyte adhering to VEC, which play an important role in the occurrence and the development of

atherosclerosis. Therefore, it is feasible that diamide was used for inducing VEC lipid peroxidation injury because the mechanism of lipid peroxidation injury induced by diamide is consistent with the mechanism of lipid peroxidation injury in vivo.

NO is produced from L-arginine by NOS [6]. There are three isoforms of NOS, namely eNOS, neural constitutive nitride oxide synthase (nNOS), and iNOS responsible for NO biosynthesis [6]. The nNOS and the eNOS are calcium/calmodulin dependent and maintain the basal physiological level of NO. The iNOS, which are not calcium/calmodulin dependent, remain unexpressed until induced by inflammatory factor or endotoxin. NO would be produced at high level when the iNOS express [6,7]. NO is a two-blade sword which plays a protective or toxic role in cells and what role NO plays depends on when, where does NO form and the type of NOS. The eNOS are the master NO synthases in VEC. But now, it was found that there is expression of iNOS in VEC.

In the control group, a little NO was produced in VEC to maintain VEC well-balanced function stably. When VEC was treated with 0.5 ml/l diamide, NO production in VEC had a declining trend with the lapse of time (see Fig. 1). At the same time, VEC were injured slightly (see Figs. 2 and 4). So it was concluded that the degree of lipid peroxidation injury induced by low concentration diamide was slight and low concentration diamide could have inhibited the expression of eNOS at a certain extent. But when VEC were treated with 5 ml/l diamide, NO production in VEC was quite dissimilar compared with that when VEC were treated with 0.5 ml/l diamide. After VEC were incubated with diamide for 4 h, NO production was far lower than that of the control group. But with the lapse of time, NO production in VEC increased sharply (see Fig. 1) and VEC were injured severely (see Figs. 2 and 3). So it was concluded that the degree of lipid peroxidation injury induced by high concentration diamide was severe and high concentration diamide could have triggered expression of iNOS, which leaded VEC to produce a great lot of NO derived from iNOS. Moreover, a great lot of NO derived from iNOS aggravated VEC lipid peroxidation injury in reverse. It was shown that too high concentration diamide could induce the expression of iNOS and did not adapt to the modeling of VEC lipid peroxidation injury. But more experiments will be required to make sure whether high concentration diamide cold induce the expression of iNOS in VEC or not and to illustrate the mechanism of iNOS' expression induced by diamide.

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