17-β-雌二醇及白黎蘆醇在心臟血管系統的抗氧化作用及機轉

Antioxidant Effects of 17-β-estradiol and Resveratrol on Cardiovascular System

中文摘要

血管內皮細胞的功能異常，平滑肌細胞及心臟纖維母細胞的增生會引起心臟血管重塑，來影響心臟血管系統的功能及結構，而引起心臟衰竭。以細胞學的角度來看，以上的過程會產生包括心血管細胞生長、細胞凋亡、細胞游走、以及發炎與纖維反應。多項因素會影響心血管細胞的變化，其中又以血管收縮素Ⅱ（angiotensinⅡ）的影響最爲重要，血管收縮素Ⅱ是藉由與其血管收縮素Ⅱ之Ⅰ型受體結合而產生一系列生理或病理的反應。有越來越多的證據顯示，血管收縮素Ⅱ產生心血管病變是藉由 NADPH 氧化酶活化形成之活性氧（reactive oxygen species）所引起。活性氧在細胞內扮演第二訊息傳遞者的角色，來調節下游的訊息傳導及轉錄因子的活性，例如：mitogen-activated protein kinase、以及 activator protein-1 (AP-1) ，而當此訊息傳導被激化後，會增加內皮素 (endothelin-1) 的分泌來調節內皮細胞的功能，血管平滑肌細胞之生長及游走，以及細胞外物質如纖維母細胞的變更。活性氧藉由改變細胞內氧化還原反應來調節訊息傳導，在正常的生理情況下此作用爲維持心血管功能的正常，但是在病理狀況下活性氧藉由氧化損傷（oxidative damage）來造成心血管功能的失調。以下三個相關實驗的主要目的是研究活性氧由血管收縮素Ⅱ激化內皮素在心血管細胞內的作用機轉，並且藉由兩種天然抗氧化劑 17-β-雌二醇 (17-β-estradiol) 及白黎蘆醇 (resveratrol) 抑制其作用機轉而降低心血管細胞之傷害。

第一部份的研究是評估由血管收縮素Ⅱ引起內皮素基因的表現是否經由活性氧的媒介而來，並且探討其在血管內皮細胞內的作用機轉。培養基的內皮細胞在接受血管收縮素Ⅱ的刺激下，以北方墨點方法(Northern blotting) 以及促進子活性分析法 (promoter activity assay) 來測內皮素基因的表現。在先接受抗氧化劑治療之內皮細胞，經由血管收縮素Ⅱ刺激下所引起 extracellular signal-regulated kinase（ERK）的磷酸化會顯著降低。由血管收縮素Ⅱ引起之內皮素基因的表現可被血管收縮素Ⅰ型受體拮抗劑（irbesartan）以及各種抗氧化劑所抑制。而 irbesartan 以及各種抗氧化劑亦可抑制由血管收縮素Ⅱ所促進細胞內活性氧的產生，並且本實驗證實由血管收縮素Ⅱ引起之 ERK 磷酸化，亦會顯著的被一些抗氧化劑所抑制。另外 ERK 的拮抗劑 U0126 可完全抑制由血管收縮素Ⅱ引起之內皮素基因的表現。將 Ras，Raf 以及 MEK1（ERK kinase）的 dominant negative mutants 一起轉染則會降低由血管收縮素Ⅱ引起之內皮素促進子活性，由此可推論 Ras-Raf-ERK pathway 參予血管收縮素Ⅱ引起內皮素的基因表現。抗氧化劑可抑制由血管收縮素Ⅱ引起之 AP-1 的活性，在變

異分析中顯示由血管收縮素Ⅱ引起內皮素基因表現中，內皮素基因之促進子(promoter) 有 cis-acting element AP-1 之結合位置。由以上實驗顯示，活性氧參予血管內皮細胞中由血管收縮素Ⅱ引起之內皮素基因的表現，並且氧化還原反應(redox-sensitive)之 ERK-mediated AP-1 轉錄路徑在血管收縮素Ⅱ引起內皮素基因表現中佔有重要角色。

第二部份的研究是評估 17-β-雌二醇（17-β-estradiol）在心臟纖維母細胞中是否會抑制由血管收縮素Ⅱ引起之細胞增生與內皮素基因表現以及其訊息傳導路徑。培養基的心臟纖維母細胞先給予 17-β-雌二醇處理之後再以血管收縮素Ⅱ刺激來檢試【3H】thymidine incorporation 以及內皮素基因的表現，並且探討 17-β-雌二醇在血管收縮素Ⅱ引起之 NADPH oxidase 活性，活性氧的形成，以及 ERK 的磷酸化。17-β-雌二醇（1-100nM）可抑制由血管收縮素Ⅱ引起之細胞 DNA 合成，但是 17-α-雌二醇不具此抑制功能。而此作用可被雌激素受體拮抗劑 ICI 182.780 所拮抗。另外 17-β-雌二醇可以抑制由血管收縮素Ⅱ引起 NADPH oxidase 活性的增加，活性氧形成的增加，ERK 磷酸化反應增加，以及 AP-1 mediated reporter 活性的增加。總言之，本實驗證實 17-β-雌二醇具備抗氧化作用可抑制由血管收縮素Ⅱ引起之細胞增生及內皮素基因的表現，以及抑制其訊息傳導路徑，由此可證實 17-β-雌二醇在血管系統的益處。

第三部份的研究是評估白黎蘆醇在血管平滑肌細胞中是否會改變由血管收縮素Ⅱ引起之細胞增生與內皮素基因表現及其訊息傳導路徑。培養基的動脈平滑肌細胞先給予白黎蘆醇的處理，再在血管收縮素Ⅱ刺激下檢試【3H】thymidine incorporation 及內皮素基因的表現。並且檢驗由血管收縮素Ⅱ引起之 ERK 磷酸化的程度來探討白黎蘆醇抑制在細胞內引起細胞增生及內皮素基因表現的機轉。在北方墨點方法以及促進子活性分析法的檢驗下，白黎蘆醇（1-100μM）可以抑制由血管緊縮素Ⅱ引起之細胞 DNA 合成及內皮素基因的表現。利用檢驗 2’7’-dichlorodihydrofluorescein diacetate (a redox sensitive fluorescent dye) 的方式測得白黎蘆醇可抑制由血管收縮素Ⅱ引起之細胞內活性氧的形成。並且白黎蘆醇以及其他抗氧劑 N-acetyl-cysteine 可抑制由血管收縮素Ⅱ引起 ERK 磷酸化以及 AP-1 的活性。總言之，本實驗證實白黎蘆醇具備抗氧化作用可抑制由血管收縮素Ⅱ引起之細胞增生及內皮素基因的表現，以及抑制其訊息傳導路徑。由此可證實白黎蘆醇在心血管系統的正面角色。

以上實驗證實血管收縮素Ⅱ引起細胞內活性氧活化並且藉由氧化還原反應之 ERK-mediated AP-1 transcriptional pathway 而促進細胞增生及內皮素基因的表現。抗氧化劑 17-β-雌二醇以及白黎蘆醇具備抑制以上所述之功能，此證據可強力支持 17-β-雌二醇以及白黎蘆醇在心血管系統的抗氧化作用

英文摘要

Diseases such as hypertension, coronary atherosclerosis, myocardial infarction

leading to heart failure are associated with cardiovascular system functional and structural changes. These include endothelial dysfunction, smooth muscle cell and cardiac fibroblast proliferation which resulting in cardiovascular remodeling. Cellular events underlying these processes involve changes in cardiovascular cells growth, apoptosis, migration, inflammation, and fibrosis. Many factors influence cellular changes, of which angiotensin II (Ang II) appears to be the most important. The physiological and pathophysiological actions of Ang II are mediated primarily via the Ang II type 1 receptor. Growing evidence indicates that Ang II induces its pleiotropic cardiovascular effects through NADPH-driven generation of reactive oxygen species (ROS). ROS function as important intracellular and intercellular second messengers to modulate many downstream signaling transduction and transcriptional factors, such as mitogen-activated protein kinase and activator protein-1 (AP-1). Induction of this signaling cascades leads to increases such as endothelin-1 (ET-1) gene expression and regulation of endothelial function, vascular smooth muscle cell growth and migration, and modification of extracellular matrix. ROS influence signaling molecules by altering the intracellular redox state. In physiological conditions, these events play an important role in maintaining cardiovascular function and integrity. Under pathological conditions ROS contribute to cardiovascular dysfunction and remodeling through oxidative damage.

The following three studies focus on related issues. The first study investigated ROS in Ang II increases ET-1 gene expression and related intracellular mechanism in cardiovascular cells. Both of the subsequent two studies pursued the relationship of two natural antioxidants, one examining 17-β-estradiol and the other study resveratrol, and the mechanisms by which they both singularly contribute to suppressing the signaling pathways and the ensuring beneficial antioxidant effect on the cardiovascular system.

In the first part of the studies, we evaluated whether ROS are involved in Ang II-induced ET-1 gene expression, and the related intracellular mechanisms occurring within vascular endothelial cells. Cultured endothelial cells were stimulated with Ang II, and Northern blotting and a promoter activity assay examined the so-elicited ET-1 gene expression. Antioxidant pre-treatment of endothelial cells was performed prior to Ang II-induced extracellular signal-regulated kinase (ERK) phosphorylation in order to elucidate the redox-sensitive pathway for ET-1 gene expression. The ET-1 gene was induced with Ang II which was inhibited with AT1 receptor antagonist (irbesartan). Ang II-enhanced intracellular ROS levels were inhibited by irbesartan and several antioxidants, and antioxidants suppressed Ang II-induced ET-1 gene expression. Furthermore, Ang II-activated ERK phosphorylation was also significantly inhibited by certain antioxidants. An ERK inhibitor, U0126 inhibited Ang II-induced ET-1

expression completely. Co-transfection of the dominant negative mutant of Ras, Raf and MEK1 (ERK kinase) attenuated the Ang II-enhanced ET-1 promoter activity, suggesting that the Ras-Raf-ERK pathway is required for the Ang II-induced ET-1 gene expression. Ang II-induced AP-1 reporter activities were inhibited by antioxidants. Moreover, mutational analysis of the ET-1 gene promoter showed that the AP-1 binding site was an important cis-acting element in Ang II-induced ET-1 gene expression. Our first data suggest that ROS are involved in Ang II-induced ET-1 gene expression within endothelial cells. The redox-sensitive ERK-mediated AP-1 transcriptional pathway plays an important role in Ang II-induced ET-1 gene expression.

In the second part of the studies we examine whether 17-β-estradiol may alter Ang II-induced cell proliferation and identify the putative underlying signaling pathways in rat cardiac fibroblasts. Cultured rat cardiac fibroblasts were pre-incubated with 17-β-estradiol then stimulated with Ang II, [3H]thymidine incorporation and ET-1 gene expression were examined. The effect of 17-β-estradiol on Ang II-induced NADPH oxidase activity, ROS formation, and ERK phosphorylation were tested to elucidate the intracellular mechanism of 17-β-estradiol in proliferation and ET-1 gene expression. Ang II increased DNA synthesis, which was inhibited with 17-β-estradiol (1–100 nM). 17-β-estradiol, but not 17-a-estradiol, inhibited the Ang II-induced ET-1 gene expression as revealed by Northern blotting and promoter activity assay. This effect was prevented by co-incubation with the estrogen receptor antagonist ICI 182.780 (1 μM). 17-β-estradiol also inhibited Ang II-increased NADPH oxidase activity, ROS formation, ERK phosphorylation, and AP-1-mediated reporter activity. In summary, our second results suggest that 17-β-estradiol inhibits Ang II-induced cell proliferation and ET-1 gene expression, partially by interfering with the ERK pathway via attenuation of ROS generation. Thus, this study provides important new insight regarding the molecular pathways that may contribute to the proposed beneficial effects of estrogen on the cardiovascular system.

In the third part of the studies, we examine whether resveratrol alters Ang II-induced cell proliferation and ET-1 gene expression and to identify the putative underlying signaling pathways in rat aortic smooth muscle cells. Cultured rat aortic smooth muscle cells were preincubated with resveratrol then stimulated with Ang II, after which [3H] thymidine incorporation and ET-1 gene expression were examined. The intracellular mechanism of resveratrol in cellular proliferation and ET-1 gene expression was elucidated by examining the phosphorylation level of Ang II-induced ERK. The inhibitory effects of resveratrol (1–100 μM) on Ang II-induced DNA synthesis and ET-1 gene expression were demonstrated with Northern blot and promoter activity assays. Measurements of 2’7’-dichlorodihydrofluorescin diacetate, a

redox sensitive fluorescent dye, showed a resveratrol-mediated inhibition of intracellular ROS generated by the effects of Ang II. The inductive properties of Ang II and H2O2 on ERK phosphorylation and AP-1-mediated reporter activity were found reversed with resveratrol and antioxidants such as N-acetyl-cysteine. In summary, Our third results speculate that resveratrol inhibits Ang II-induced cell proliferation and ET-1 gene expression, which involves the disruption of the ERK pathway via attenuation of ROS generation. Thus, this study provides important insight into the molecular pathways that may contribute to the proposed beneficial effects of resveratrol on the cardiovascular system.

In conclusion, our three in vitro studies all clearly indicate that Ang II-induced intracellular ROS act as second messengers and via redox-sensitive ERK-mediated AP-1 transcriptional pathway to stimulate the cell proliferation and ET-1 expression in various cardiovascular cells. Both 17-β-estradiol and resveratrol have inhibitory effects on this signaling. These data strongly support the proposed beneficial antioxidant effects of 17-β-estradiol and resveratrol in the cardiovascular system.