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研究论文

血管紧张素Ⅱ受体阻断对心肌成纤维细胞信号转导相关基因表达谱的影响

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摘 要：为了研究血管紧张素Ⅱ (angiotensin II, Ang II)受体在成年大鼠心肌成纤维细胞的信号转导机制，分离及培养成年Sprague-Dawley大鼠心肌成纤维细胞，采用免疫组化染色测定Ang Ⅱ受体的蛋白表达。将细胞随机分为四组进行药物干预48 h：Ang Ⅱ组、Ang Ⅱ + losartan 组、Ang Ⅱ + PD123319 组和Ang Ⅱ + losartan + PD123319 组。抽提mRNA制备cDNA探针，与G蛋白耦联受体信号通路发现者基因芯片杂交，筛选表达差异的基因。发现血管紧张素Ⅱ 1 型(angiotensin II type 1, AT1)受体被losartan阻断后，Ang Ⅱ刺激的心肌成纤维细胞血管紧张素Ⅱ 2 型(angiotensin II type 2, AT2)受体蛋白高表达；34 个基因表达差异在2倍以上，30 个下调，4 个上调，其最大改变不超过20倍；9 条信号通路被活化：cAMP/PKA、Ca^{2+}、PKC、PLC、MAPK、PI-3K、NO-cGMP、Rho、NF-κB 通路。当AT2 受体被PD123319 阻断时，64 个基因表达差异在2倍以上，48 个下调，16 个上调；11 条途径基础活化，其中7 个基因的改变在30 倍以上：Cyp19a1 (37 倍)、Il1r2 (42 倍)、Cflar (53 倍)、Bcl21 (31 倍)、Pik3cg (278 倍)、Cdkn1a (90 倍)、Agt (162 倍)。在AT1 受体阻断的基础上再阻断AT2 受体，46 个基因表达差异在2倍以上，36 个下调，10 个上调；11 条信号途径全部活化。其结果与单独阻断AT2 受体信号途径基本一致。RT-PCR 选取IL-1β 和TNF-α 进行验证，结果与芯片各组间的变化趋势基本相符。结果表明，在成年大鼠心肌成纤维细胞，AT2 受体阻断明显不同于AT1 受体阻断，在信号转导通路相关基因表达谱上，两者有显著差异。

关键词：心肌成纤维细胞；AT2 受体；cDNA 芯片
中图分类号：R363.2+1

Alteration of signal transduction-associated gene expression in rat cardiac fibroblasts induced by blocking angiotensin II receptors

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Abstract: To investigate the molecular mechanism of angiotensin II (Ang II) receptor activation in adult rat cardiac fibroblasts, the expressions of cell signal transduction-associated genes were studied by using cDNA microarray. Cardiac fibroblasts of adult Sprague-Dawley rats (230~250 g) were isolated and cultured. The cells were divided into 4 groups: Ang II, Ang II + losartan, Ang II + PD123319, Ang II + losartan + PD123319. The expressions of Ang II receptors were studied by immunohistochemical staining. Total RNA was extracted and purified. After cDNA synthesis and biotin-16-dUTP labeling, the probes were denatured and hybridized with GEArray Q Series mouse G Protein-coupled Receptors Signaling Pathway Finder Gene Array (MM-025) containing 96 genes associated with 11 pathways. The arrays were scanned with a Uniscand1000 scanner and further analyzed with GEArray Analyzer software. RT-PCR was used to further confirm the results of gene microarray. The results of immunohistochemical staining showed that the expression of Ang II type 2 (AT2) receptor was evidently induced by Ang II stimulation when Ang II type 1 (AT1) receptor was blocked. The results of gene array indicated that blocking AT1 receptor changed 34 genes (more than 2 folds), 30 were down-regulated and 4 were up-

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regulated. The maximum change was not beyond 20 folds. The following 9 pathways were activated: cAMP/PKA, Ca^{2+}, PKC, PLC, MAPK, PI-3 kinase, NO-cGMP, Rho, NF-κB pathways. Blockade of AT2 receptor caused 64 genes changing more than 2 folds (48 were down-regulated and 16 were up-regulated). Eleven pathways were basically activated. The change of the following 7 genes was over 30 folds: Cyp19a1 (37 folds), Il1r2 (42 folds), Cflar (53 folds), Bcl21 (31 folds), Pik3cg (278 folds), Cdkn1a (90 folds), Agt (162 folds). According to the activated extent, the signal transduction pathways in turn were PI-3 kinase, NF-κB and JAK-STAT pathways. Blocking both AT1 and AT2 receptors changed 46 genes more than 2 folds (36 were down-regulated and 10 were up-regulated). Eleven pathways were basically activated. The results of RT-PCR of IL-1β and TNF-α confirmed the observations in gene microarray. Our results show that Ang II can induce a high expression of AT2 receptor in adult rat cardiac fibroblasts when AT1 receptor is blocked, and the signal mechanism of AT2 receptor is clearly different from that of AT1 receptor.

Key words: cardiac myoblasts; receptor, angiotensin, type 2; cDNA microarray

研究表明，心肌肥厚及慢性心衰的主要病理生理机制是进行性发展的心肌重塑(cardiac remodeling)。心肌重塑主要表现为心肌组织细胞表型改变、心肌细胞肥大、成纤维细胞增生和胞外基质蛋白(胶原纤维)沉积。研究证实心肌内源性肾素-血管紧张素系统(renin-angiotensin system, RAS)在心肌重塑的发生、发展中起着重要的调控作用[1]。血管紧张素Ⅱ(angiotensin II, Ang II)是RAS主要的活性物质，主要通过AT1和AT2受体(angiotensin II type 1 and 2 receptors)发挥效应[2]。研究发现Ang Ⅱ可促使心肌成纤维细胞(cardiac fibroblasts, CFs)增殖，合成分泌胶原等细胞外基质成分，介导间质纤维化而参与心肌重塑[3]。Ang Ⅱ刺激CFs还可分泌多种细胞因子，这些因子以旁分泌或自分泌的方式调控CF自身和周围的心肌细胞代谢结构功能而参与心肌重塑[4]。Ang Ⅱ对CFs介导的多种生物学效应，目前认为是由AT1受体介导。临床应用AT1受体拮抗剂防治高血压和心衰已取得较好的近期疗效。但在AT1受体被抑制的同时，必将使AT2受体的作用增强，然而AT2受体的作用及分子机制目前尚未阐明[5,6]。有文献报道AT2受体具有与AT1受体相拮抗的生长抑制效应，在心肌梗死中具有心肌保护作用。然而AT2受体在心肌肥大及重塑中的作用，目前尚未定论。我们在本室多年对Ang Ⅱ及心肌重塑研究的基础上，采用基因芯片的方法，研究不同受体阻断后CFs信号通路基因表达谱的改变，探求AT2受体在CFs的信号转导机制，寻找成年大鼠CFs中AT2和AT1受体的差异。

1 材料与方法

1.1 材料 雄性Sprague-Dawley (SD)大鼠，体重230~250 g，由西安交通大学医学院实验动物中心提供。胶原酶Ⅰ、胰蛋白酶、Ang Ⅱ和PD123319 (Sigma公司); losartan (Du Pont & Merk Pharmaceuticals公司); TRIzol、DMEM (Gibco公司); MMLV反转录酶、RNase抑制剂、逆转录试剂盒、*Taq*聚合酶、dNTP和PCR试剂(Promega公司); 生物素-16-dUTP购自Roche公司；芯片系美国Superarray公司GEArray Q系列小鼠G蛋白耦联受体信号通路发现者基因芯片(MM-025)，芯片的基因分布图及相关信息可从公司的网站检索到(www.superarray.com); 兔抗大鼠AT2多抗(武汉博士德公司)。

1.2 方法

1.2.1 成年SD大鼠CFs的分离及培养 采用本室已建立的胶原酶Ⅰ、胰蛋白酶消化及差速贴壁的方法[3]分离培养原代CFs。培养条件为37℃、5% CO_2。细胞在含20%胎牛血清的DMEM中培养至亚融合状态，经12 h无血清预适应后，进行药物干预。

1.2.2 免疫组化染色测定AT2受体蛋白表达 将细胞培养在玻片上，分为对照组、Ang Ⅱ (1×10^{-6} mol/L)组、losartan (1×10^{-5} mol/L)组、Ang Ⅱ (1×10^{-7} mol/L) + losartan (1×10^{-5} mol/L)组、Ang Ⅱ 1×10^{-6} mol/L) + losartan (1×10^{-5} mol/L)组、Ang Ⅱ(1×10^{-5} mol/L) + losartan (1×10^{-5} mol/L)组。药物干预作用48 h，进行常规免疫组化操作(ABC法)。即以抗生物素的过氧化物酶反应检测，依次加入兔抗大鼠AT2多克隆抗体(一抗)、生物素标记的羊抗兔IgG (二抗)、DAB显色、复染和封片。

1.2.3 基因芯片检测Ang Ⅱ受体阻断后的信号分子表达变化

1.2.3.1 药物干预 细胞随机分为四组，进行药物干预Ang Ⅱ (1×10^{-5} mol/L)、Ang Ⅱ (1×10^{-5} mol/L) + losartan (1×10^{-5} mol/L)、Ang Ⅱ (1×10^{-5} mol/L) +

PD123319 (1×10^{-5} mol/L)、Ang Ⅱ (1×10^{-5} mol/L) + losartan (1×10^{-5} mol/L) + PD123319 (1×10^{-5} mol/L)，药物作用 48 h 后收集细胞。losartan 是 AT1 受体特异性阻断剂，PD123319 是 AT2 受体特异性阻断剂。

1.2.3.2 细胞总 RNA 的抽提和测定 按 TRIzol 一步法抽提细胞总 RNA，测量其 260 nm 和 280 nm 下的光吸收值，并计算其比值以及溶液浓度。

1.2.3.3 探针标记 先将 GEArray 标记混合液 42℃孵育 1 min，加 10 μl 到 42℃孵育的退火混合物 10 μl (含总 RNA 5 μg)中，混匀，42℃ 90 min。标记反应液(10 μl/样品)含标记混合液 4 μl、生物素 -16-dUTP 2 μl、RNase 抑制剂 1 μl、MMLV 反转录酶 1 μl 和无 RNase 的纯水 2 μl。将标记好的 cDNA 探针溶液置于 94℃预变性 5 min，迅速冰浴冷却待用。

1.2.3.4 杂交 加5 ml去离子水至杂交管将芯片膜完全湿润，将水弃去；加 2 ml 60℃预热的预杂交液，60℃下 6 r/min 预杂交 1.5 h；加已变性的 cDNA 探针入预杂交液，60℃下 6 r/min 杂交过夜。洗膜。

1.2.3.5 化学发光法检测芯片杂交结果 加 1.5 ml 的 GEAb 封闭液 Q 入杂交管，室温下孵育 40 min；去除封闭液，加 2 ml 稀释的 AP 结合缓冲液，温和振荡 10 min； 洗膜 4 次；加 1.0 ml CDP-Star 化学发光底物入杂交管，室温下静置 2 min，检测芯片的杂交结果。取出芯片，用 X- 射线胶片曝光。

1.2.3.6 芯片数据分析 用紫光 uniscan d1000 扫描仪扫描芯片，获取基因表达的信号强度值，用 Scanalyzer 软件对扫描图像进行数字化处理，用芯片配套软件GEArray Analyzer对获取的原始信号进行均衡和修正。用内参照基因(β-actin、GAPDH、cyclophilin A 和 RPL13A)进行校正，并对每个基因的 4 个重复点进行平均。然后进行样品间基因表达丰度的分析。判断基因差异表达的筛选标准：比值>2.0 (上调 2 倍)或<0.5 (下调 2 倍) 。

1.2.4 半定量RT-PCR 采用TRIzol一步法提取细胞中的 RNA，以 5 μg 总 RNA 进行逆转录合成 cDNA，用不同引物分别进行 30 个循环的扩增(94℃，1 min；退火温度，1 min；72℃，1 min); 72℃延伸 5 min。引物的设计采用 Primer 5 软件，序列及各对引物的退火温度如表 1。PCR 产物取 8 μl，在 2% 琼脂糖凝胶上电泳，用凝胶成像系统对条带灰度进行分析。以目的基因与内参照 β-actin 的光密度(optical density)比值作为半定量指标。

1.3 统计学分析 数据以 mean±SD 表示，组间比较采用 *t* 检验，$P<0.05$ 认为有统计学差异。

2 结果

2.1 免疫组化结果

在对照组、Ang Ⅱ (1×10^{-6} mol/L)组、losartan (1×10^{-5} mol/L)组和 Ang Ⅱ (1×10^{-7}, 1×10^{-6}, 1×10^{-5} mol/L) + losartan (1×10^{-5} mol/L)组，仅在最后一组 Ang Ⅱ浓度变为 1×10^{-5} mol/L 时 AT2 受体才呈现高表达(图 1)，其余各组均没有 AT2 受体表达，呈阴性结果(结果未示)。光镜下 AT2 受体的阳性染色呈淡棕色、条状分布，主要位于 CFs 膜上。

2.2 细胞总 RNA 质量

Ang Ⅱ处理组 OD_{260}/OD_{280} 为 1.83、Ang Ⅱ + losartan 组 OD_{260}/OD_{280} 为 1.82、Ang Ⅱ + PD123319 组 OD_{260}/OD_{280} 为 1.94、Ang Ⅱ + losartan + PD123319 组 OD_{260}/OD_{280} 为 1.87。它们与电泳分析结果共同表明，本实验得到了高纯度的 RNA，完全符合实验要求。

2.3 基因芯片结果

Ang Ⅱ处理组、Ang Ⅱ + losartan 组、Ang Ⅱ + PD123319 组、Ang Ⅱ + losartan + PD123319 组芯片图像结果见图 2。

表 1. TNF-α、IL-1β 和 β-actin 的引物序列和退火温度

Table1. Primer sequences and corresponding annealing temperature

Gene	Primer	Annealling temperature (ºC)	Length (bp)
β-actin	5’ CCTGTACGCCAACACAGTGC 3’	57	211
	5’ ATACTCCTGCTTGCTGATCC 3’		
TNF-α	5’ TGGTATGAAGTGGCAAATCG 3’	53	327
	5’ TCCCAACAAGGAGGAGAAGT 3’		
IL-1β	5’ CTTCTTTGGGTATTGTTTGG 3’	51	304
	5’ TGATGACGACCTGCTAGTGT 3’		

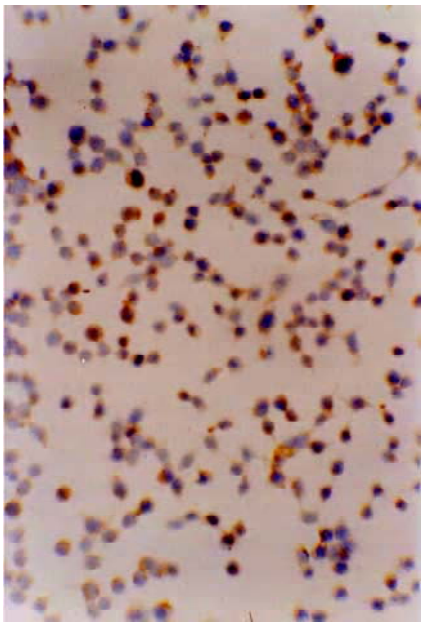

图 1. AT2 受体的阳性染色

Fig.1. Positive staining of AT2 receptor in cardiac fibroblasts was only observed when treated by Ang II (1×10^{-5} mol/L) + losartan (1×10^{-5} mol/L).

Ang Ⅱ + losartan 组与 Ang Ⅱ 组比较显示 AT1 受体阻断后，34 个基因表达差异在 2 倍以上(表 2)，其中 30 个下调，4 个上调。其最大改变不超过 20 倍。9 条信号通路被活化：cAMP/PKA (Rgs2、Vegfa、Ptgs2); Ca^{2+} (Elk4、Ccl2、Ccl4); PKC (Jun、Mmp9、Myc、Nos2、Npy、Agtr2); PLC (Icam1); MAPK (Fgf2、Ccl2); PI-3K (Akt1); NO-cGMP (Tnf、RIKEN cDNA 4933430F08 gene); Rho (Ctgf、Il1β); NF-κB (Csf3、Il2)通路。Ang Ⅱ + PD123319 组与 Ang Ⅱ 组比较显示 AT2 受体阻断后，64 个基因表达差异在 2 倍以上(表 3)，48 个下调，16 个上调。11 条途径基础活化，其中 7 个基因的改变在 30 倍以上：Cyp19a1 (37 倍)、Il1r2 (42 倍)、Cflar (53 倍)、Bcl21 (31 倍)、Pik3cg (278 倍)、Cdkn1a (90 倍)、Agt (162 倍)。Ang Ⅱ + losartan + PD123319 组与 Ang Ⅱ + losartan 组比较显示在 AT1 受体阻断的基础上再阻断 AT2 受体后，46 个基因表达差异在 2 倍以上，36 个下调，10 个上调。11 条途径基础活化。其中除 Ptgs2 (235 倍)、Agtr2 (81 倍)、Il1β (8 倍)、Tnf (7 倍)，其余改变均在 3 倍左右。

2.4 RT-PCR 鉴定结果

我们选取2个感兴趣的基因IL-1β和TNF-α作RT-PCR 验证。IL-1β 和 TNF-α 的 RT-PCR 产物凝胶电泳图分别见图 3、4。 芯片检测结果表明，IL-1β 在 Ang Ⅱ 组、Ang Ⅱ + losartan 组、Ang Ⅱ + PD123319 组、Ang Ⅱ + losartan + PD123319 组的表达水平分别是 2.209E-2、7.068E-3、4.225E-2、8.450E-4。AT1 受体阻断，IL-1β 下调 3.125 倍；AT2 受体阻断，IL-1β 上调 1.9 倍，共阻断下调 26 倍。RT-PCR 显示：AT1 受体阻断，IL-1β 下调 5%；AT2 受体

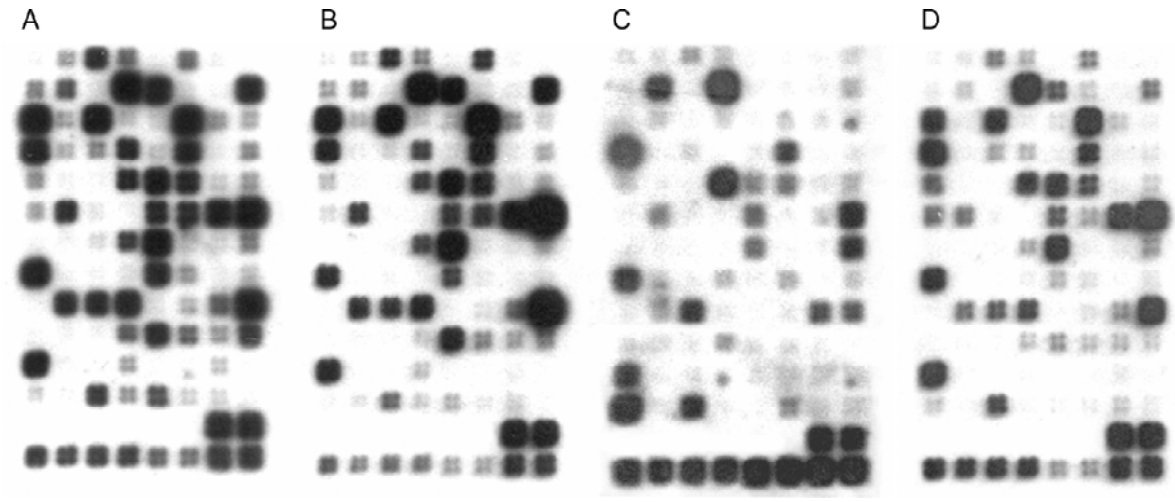

图 2. 芯片图像

Fig.2. Images of gene chips. *A*: Ang II group. *B*: Ang II + losartan group. *C*: Ang II + PD123319 group. *D*: Ang II + losartan + PD123319 group.

表 2. Losartan 阻断 AT1 受体后差异表达基因

Table 2. Differentially expressed genes induced by blockade of AT1 receptor by losartan

GenBank	Symbol	Description	Expression ratio (Ang II + losartan/Ang II)
NM_007420	Adrβ2	Adrenergic receptor, beta 2	4.920E-1
NM_009652	Akt1	Thymoma viral proto-oncogene 1	4.228E-1
NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	2.169E-1
NM_010217	Ctgf	Connective tissue growth factor	4.200E-1
NM_010336	Edg2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	4.067E-1
NM_010101	Edg3	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	3.311E-1
NM_007923	Elk4	ELK4, member of ETS oncogene family	2.484E-1
NM_008006	Fgf2	Fibroblast growth factor 2	1.214E-1
NM_010323	Gnrhr	Gonadotropin releasing hormone receptor	5.567E-2/0
NM_016976	Gprc1a	Mus musculus G-protein-coupled receptor, family C, group 1, member A	3.846E-1
NM_008174	Grm8	Glutamate receptor, metabotropic 8	1.940E-1
NM_173372	Grm6-ESTs	ESTs, Glutamate receptor, metabotropic 6	4.529E-1
NM_010493	Icam1	Intercellular adhesion molecule	5.099E-2
NM_008361	Il1β	Interleukin 1 beta	3.200E-1
NM_008366	Il2	Interleukin 2	1.675E-1
NM_010591	Jun	Jun oncogene	7.501E-2
NM_013582	Lhcgr	Luteinizing hormone/choriogonadotropin receptor	4.056E-1
NM_145429	Arrβ2	Arrestin, beta 2	3.517E-1
NM_013599	Mmp9	Matrix metalloproteinase 9	6.818E-2
NM_010849	Myc	Myelocytomatosis oncogene	2.574E-1
NM_010927	Nos2	Nitric oxide synthase 2, inducible, macrophage	1.217E-1
NM_023456	Npy	Neuropeptide Y	2.768E-1
NM_008962	Ptgdr	Prostaglandin D receptor	1.909E-2
NM_030701	Pumag-pending	Mus musculus interferon-gamma inducible gene, Puma-g (Pumag-pending)	7.358E-2
NM_009061	Rgs2	Regulator of G-protein signaling 2	1.866E-1
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	1.673E-1
NM_013693	Tnf	Tumor necrosis factor	3.926E-1
NM_011648	Tshr	Thyroid stimulating hormone receptor	2.681E-1
NM_009463	Ucp1	Uncoupling protein 1, mitochondrial	3.437E-1
NM_009505	Vegfa	Vascular endothelial growth factor A	2.362E-1
NM_028967	4933430F08Rik	RIKEN cDNA 4933430F08 gene	2.696E+0
NM_007429	Agtr2	Angiotensin II receptor, type 2	1.982E+1
NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2	2.128E+0
NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	6.647E+0

阻断，IL-1β 上调 37%，共阻断下调 46% (P<0.05)。TNF-α 在四组的表达水平是 2.143E-1、8.415E-2、4.587E-2、1.232E-2。AT1 受体阻断，TNF-α 下调 2.55 倍；AT2 受体阻断，TNF-α 下调 4.67 倍，共阻断下调 17 倍。 RT-PCR 显示：AT1 受体阻断，TNF-α 下调 26%；AT2 受体阻断，TNF-α 下调 37%，共同阻断下调 44% (P<0.05)。RT-PCR 验证结果与芯片的筛查结果趋势基本相符。

表 3. PD123319 阻断 AT2 受体后差异表达基因

Table 3. Differentially expressed genes induced by blockade of AT2 receptor by PD123319

GenBank	Symbol	Description	Expression ratio (Ang II + PD/Ang II)
NM_013506	Eif4a2	Eukaryotic translation initiation factor 4A2	3.474E-1
NM_009605	Acrp30	Adipocyte complement related protein of 30 kDa	2.930E-1
NM_007419	Adrβ1	Adrenergic receptor, beta 1	3.942E-1
NM_007420	Adrβ2	Adrenergic receptor, beta 2	1.024E-1
NM_007428	Agt	Angiotensinogen	6.148E-3
NM_011784	Agtrl1	Angiotensin receptor-like 1	3.943E-1
NM_009741	Bcl2	B-cell leukemia/lymphoma 2	6.676E-2
NM_009743	Bcl2l	Bcl2-like	3.287E-2
NM_007588	Calcr	Calcitonin receptor	4.456E-1
NM_007631	Ccnd1	Cyclin D1	2.173E-1
NM_007633	Ccne1	Cyclin E1	1.536E-1
NM_007669	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	1.116E-2
NM_009875	Cdkn1b	Cyclin-dependent kinase inhibitor 1B (P27)	4.783E-1
NM_009805	Cflar	CASP8 and FADD-like apoptosis regulator	1.889E-2
NM_009896	Socs1	Suppressor of cytokine signaling 1	7.762E-2
NM_007707	Socs3	Suppressor of cytokine signaling 3; cytokine inducible SH2-containing protein 3	1.135E-1
NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	3.513E-1
NM_010217	Ctgf	Connective tissue growth factor	2.621E-1
NM_007810	Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1	2.654E-2
NM_013503	Drd5	Dopamine receptor 5	1.619E-1
NM_019819	Dusp14	Dual specificity phosphatase 14	7.109E-2
NM_007901	Edg1	Endothelial differentiation sphingolipid G-protein-coupled receptor 1	1.575E-1
NM_022983	Edg7	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 7	3.667E-1
NM_053190	Edg8	Endothelial differentiation, sphingolipid G-protein-coupled,receptor8	4.416E-1
NM_007913	Egr1	Mouse early growth response 1	3.153E-1
NM_007923	Elk4	ELK4, member of ETS oncogene family	1.275E-1
NM_010171	F3	Coagulation factor III	4.999E-1
XM_132546	Ghrhr	Growth hormone releasing hormone receptor	2.392E-2
NM_008139	Gnαq	Guanine nucleotide binding protein, alpha q polypeptide	1.143E-1
NM_008174	Grm8	Glutamate receptor, metabotropic 8	1.300E-1
NM_013803	Gprc2a	G-protein-coupled receptor, family C, group 2, member A	7.051E-3
XM_149971	Grm5	Metabotropic glutamate receptor mGluR5 (AI850523)	3.747E-1
NM_173372	Grm6-ESTs	ESTs, Glutamate receptor, metabotropic 6	2.508E-2
NM_008293	Hsd3β1	Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	1.243E-1
NM_010555	Il1r2	Interleukin 1 receptor, type II	2.357E-2
NM_021283	Il4	Interleukin 4	3.568E-2
NM_008416	Junb	Mus musculus Jun-B oncogene	3.877E-1
NM_008497	Lhβ	Luteinizing hormone beta	4.829E-2
NM_013582	Lhcgr	Luteinizing hormone/choriogonadotropin receptor	4.782E-2
NM_145429	Arrβ2	Arrestin, beta 2	2.087E-1
XM_144986	Mgr8	ESTs, Moderately similar to Mgr8_mouse Metabotropic glutamate receptor 8 precursor (mGluR8)	4.491E-1
NM_023456	Npy	Neuropeptide Y	4.053E-1
NM_013622	Oprδ1	Opioid receptor, delta 1	6.704E-2
NM_011011	Oprκ1	Opioid receptor, kappa 1	1.253E-1

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GenBank	Symbol	Description	Expression ratio (Ang II + PD/Ang II)
NM_011062	Pdpk1	Mus musculus phosphoinositide-dependent protein kinase 1	8.766E-2
NM_020272	Pik3cg	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	3.593E-3
NM_013693	Tnf	Tumor necrosis factor	2.140E-1
NM_011648	Tshr	Thyroid stimulating hormone receptor	2.505E-2
NM_028967	4933430F08Rik	RIKEN cDNA 4933430F08 gene	1.300E+1
NM_009642	Agtrap	Angiotensin II, type I receptor-associated protein	1.381E+1
NM_020028	Edg4	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 4	4.150E+0
NM_010493	Icam1	Intercellular adhesion molecule	2.775E+0
NM_013599	Mmp9	Matrix metalloproteinase 9	4.031E+0
NM_010849	Myc	Myelocytomatosis oncogene	3.876E+0
NM_011164	Prl	Prolactin	2.295E+0
NM_008962	Ptgdr	Prostaglandin D receptor	8.772E+0
NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2	8.060E+0
NM_030701	Pumag-pending	Mus musculus interferon-gamma inducible gene, Puma-g (Pumag-pending)	8.312E+0
NM_145383	Rho	Rhodopsin	5.251E+1
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	1.450E+1
NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	2.949E+1
NM_009463	Ucp1	Uncoupling protein 1, mitochondrial	4.449E+0
NM_011693	Vcam1	Vascular cell adhesion molecule 1	2.545E+0
NM_009505	Vegfa	Vascular endothelial growth factor A	8.096E+0

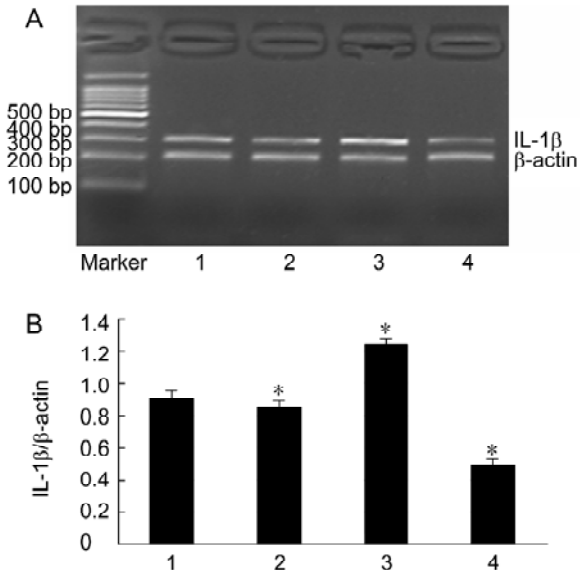

图 3. IL-1β 的 RT-PCR 产物凝胶电泳分析

Fig.3. RT-PCR product of IL-1β. *A*: Agarose gel electrophoresis of IL-1β mRNA. 1, Ang II group; 2, Ang II + losartan group; 3, Ang II + PD123319 group; 4, Ang II + losartan + PD123319 group. *B*: Quantitive analysis of IL-1β mRNA normalized to β-actin. $^{*}P<0.05$ compared with Ang II group.

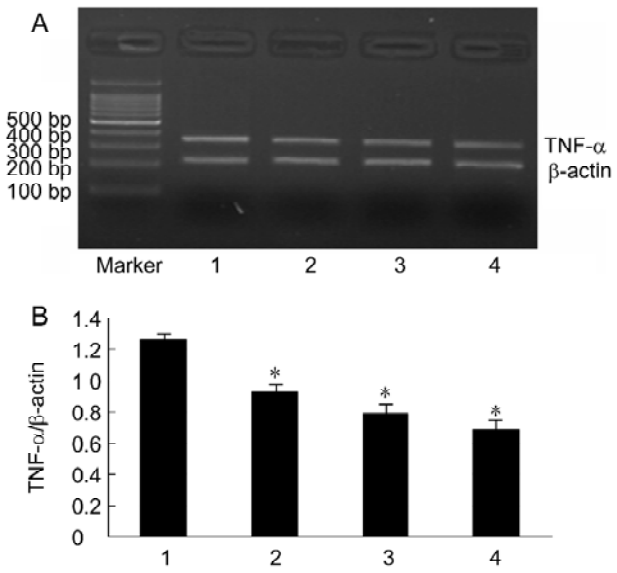

图 4. TNF-α 的 RT-PCR 产物凝胶电泳分析

Fig. 4. RT-PCR product of TNF-α. *A*: Agarose gel electrophoresis of TNF-α mRNA. 1, Ang II group; 2, Ang II + losartan group; 3, Ang II + PD123319 group; 4, Ang II + losartan + PD123319 group. *B*: Quantitive analysis of TNF-α mRNA normalized to β-actin. $^{*}P<0.05$ compared with Ang II group.

3 讨论

AT1和AT2受体均属G蛋白耦联受体。AT1受体由359个氨基酸组成，AT2受体由363个氨基酸组成，二者之间有32%~35%的同源性。AT1受体分布广泛，而AT2受体在成人机体仅限于几个器官的低水平表达(如脑、心脏等)，AT2受体在组织重塑和炎症中表达增强，蕴涵着AT2受体可能的病理意义[2]。AT2与AT1受体在心肌重构中可能介导不同的作用。文献报道在压力超负荷诱导的心肌肥厚模型中，冠状动脉的增厚和血管壁纤维化，在$Agtr2^{-}$小鼠明显加强，而在过表达小鼠显著减弱，且AT1受体阻断剂抑制纤维化的效应在$Agtr2^{-}$鼠明显减弱，说明AT2受体参与了AT1受体阻断剂介导的抑制间质纤维化效应[7]。在Wistar-Kyoto老龄鼠心肌肥厚模型中，AT2受体部分介导了AT1受体阻断剂的抗肥大效应[8]。说明AT2受体可能参与改善心肌重构的过程，然而其确切作用及机制尚不明确。本研究在基因水平对成年CFs中AT1与AT2受体阻断后的信号转导相关基因表达谱进行研究，进而探求AT2受体在CFs的信号转导机制，明确AT2和AT1受体的差异。

本研究在我室以往研究的基础上，先用免疫组化发现：Ang Ⅱ (1×10^{-5} mol/L) + losartan (1×10^{-5} mol/L)作用CFs 48 h后，AT2受体蛋白高表达。表明AT1受体阻断后，高水平的Ang Ⅱ可诱导CFs的AT2受体蛋白高表达，随后的基因芯片结果也显示AT1受体阻断后，AT2受体基因上调20倍。说明高水平的Ang Ⅱ从转录水平上调AT2受体的表达。CFs的AT2受体在蛋白质水平的高表达必将使AT2受体的作用加强，放大AT2受体的信号转导效应。

Ang Ⅱ作用于CFs上的AT1受体，可促进CFs增殖和胶原蛋白合成，介导间质纤维化而参与心肌间质重塑[9]。Ang Ⅱ对CFs的这些效应是通过CFs胞内信号途径介导的。AT1受体通过Gq蛋白激活PLC，水解PIG2生成IP3和DG2，使胞内Ca^{2+}增加，活化PKC介导生物学效应。AT1受体本身虽然不具有内源性酪氨酸激酶样活性，但与Ang Ⅱ结合后却能启动细胞内酪氨酸磷酸化的级联反应。AT1受体在与Ang Ⅱ结合后激活ERK和Ras，还可激活JNK和p38 MAPKs的信号转导通路[10]。AT1受体还可通过Gi蛋白抑制腺苷酸环化酶活性，从而抑制cAMP/PKA途径[11]。有报道在人CFs，Ang Ⅱ结合AT1受体，通过Ca^{2+}敏感性的PKC-依赖性酪氨酸激酶途径，刺激细胞蛋白质合成过程[12]。Abbasi等报道MEKK3参与AT1受体活化后的calcineurin/NFAT途径[13]。本研究结果提示：losartan阻断AT1受体后(Ang Ⅱ + losartan组与Ang Ⅱ组比较) 34个基因表达改变2倍以上，其最大改变不超过20倍。涉及9条信号通路活化：cAMP/PKA (Rgs2、Vegfa、Ptgs2); Ca^{2+} (Elk4、Ccl2、Ccl4); PKC (Jun、Mmp9、Myc、Nos2、Npy、Agtr2); PLC (Icam1); MAPK (Fgf2、Ccl2); PI-3K (Akt1); NO-cGMP (Tnf、RIKEN cDNA 4933430F08 gene); Rho (Ctgf、Il1β)，NF-κB (Csf3、Il2)通路。本研究结果表明，AT1受体在CFs的信号转导机制较为复杂，涉及多条信号转导通路的活化。各条途径之间的交互作用需要进一步研究。Seta等报道AT1受体Tyr-319的磷酸化介导Ang Ⅱ引起的表皮生长因子受体的跨活化[14]。本研究结果提示：AT1受体阻断同时调节了多种G蛋白耦联受体(Adrβ2、Edg2、Edg3、Gnrhr、Gprc1a、Grm8、Grm6、Lhcgr、Arrβ2、Ptgdr、Tshr)的表达，其详细机制有待进一步研究。

对于AT2受体的信号转导途径没有AT1受体研究得广泛。有报道心肌AT2受体通过kinin/NO机制抑制间质纤维化[15]。不同于AT1受体，Ang Ⅱ结合AT2受体并不影响细胞内cAMP水平，但在不同细胞cGMP水平会增高或降低，且cGMP水平受NO调控。Pulakat等报道AT2受体胞内第二、三个环与细胞内cGMP的水平相关[16]。AT2受体不同于AT1受体，在细胞内不活化PLC。有报道心脏AT2受体参与心脏肥大的调节，且PKC信号途径参与此作用[17]。在PC12W细胞Ang Ⅱ结合AT2受体，通过活化酪氨酸蛋白磷酸酶抑制鸟苷酸环化酶，降低细胞内cGMP水平[18]。AT2受体能激活许多磷酸酶如SHP-1、MKP-1、PP2A等。Ang Ⅱ通过AT2受体激活MKP21和PP2A活性而抑制ERK1/2[19]。有报道Ang Ⅱ刺激AT2受体后下游信号途径是calcineurin/NF-AT/eNOS途径[20]。Andresen等报道AT2-AT1受体的交互作用是由NO和RhoA机制介导[21]。CNK1蛋白参与AT2受体介导的信号转导通路[22]。本研究结果提示：PD123319阻断AT2受体后，64个基因表达差异(Ang Ⅱ + PD123319组与Ang Ⅱ组比较)，11条信号途径全部活化(cAMP/PKA、Ca^{2+}、PKC、PLC、PTK, MAPK、PI-3K、NO-cGMP、Rho、NF-κB、JAK-STAT通路)。其中

PI-3K 通路：Pik3cg (278 倍), Cflar (53 倍), Bcl21 (31 倍); NF-κB 通路：Agt (162 倍)，Il4 (28 倍); JAK-STAT 通路：Cdkn1a (90 倍)。该结果表明 AT2 受体在 CF 的信号转导机制较为复杂，涉及多条信号转导通路。其中三条途径活化程度明显高于其他途径。Nouet 等报道 AT2 受体通过 ATIP1 蛋白，抑制其他酪氨酸蛋白激酶受体，呈现出 AT2 受体与酪氨酸蛋白激酶受体的负性交互作用，从而抑制细胞生长[23]。Horiuchi 等报道 AT2 受体通过活化磷酸酶与其他 G 蛋白耦联受体的信号产生交互作用[19]。本研究结果提示 AT2 受体阻断同时调节了其他多种 G 蛋白耦联受体(Adrβ1、Adrβ2、Agtrl1、Calcr、Drd5、Edg1、Edg7、Ghrhr、Gnaq、Grm8、Gprc2a、Grm5、Grm6、Lhcgr、Arrβ2、Mgr8、Oprd1、Oprk1、Tshr)的表达，其详细的作用机制有待进一步研究。

研究表明，losartan 改善心肌缺血后的心肌结构和功能、减轻左室重构的主要机制，与其在阻断 AT1 受体的同时使心肌组织中 Ang Ⅱ水平反应性增高，刺激 AT2 受体的表达相关。在此基础上 AT2 受体的作用机制不明。losartan 阻断 AT1 受体，会使更多游离的 Ang Ⅱ与 AT2 受体结合，使 AT2 受体的作用加强。我们的研究表明 losartan 阻断 CFs 的 AT1 受体后，高水平的 Ang Ⅱ可诱导 AT2 受体蛋白高表达(机制有待进一步研究)，AT2 受体在蛋白质水平高表达将使 AT2 受体的作用加强。本研究在阻断 AT1 受体的同时阻断 AT2 受体，对信号转导相关基因表达谱进行研究，探求 AT2 受体的作用机制。结果表明在 AT1 受体阻断的基础上再阻断 AT2 受体后，CFs 的 46 个基因表达差异在 2 倍以上(Ang Ⅱ + losartan + PD123319 组与 Ang Ⅱ + losartan 组比较)，11 条信号途径全部活化。这一结果与单独阻断 AT2 受体信号途径基本一致。说明 AT2 受体活化后的信号转导途径并不象多数文献报道的集中在NO-cGMP 途径，而是多条信号途径的活化。

有报道在新生大鼠 CFs 存在 RAS 的各个组分，Ang Ⅱ负性调控 angiotensinogen 的表达，且这一作用是 AT1 受体依赖性的[24]。有研究报道 AT2 受体负性调控 RAS，抑制肾素的合成及 Ang Ⅱ的形成[25]。我们的研究表明，在基因水平 losartan 上调 angiotensinogen 的表达 1.367 倍，PD123319 下调 angiotensinogen 的表达 162.63 倍，说明 AT2 受体明显参与 angiotensinogen 表达调控。但是基因水平的表达并不能完全代表蛋白质水平的表达，在蛋白质水平 AT2 受体是否参与 angiotensinogen 的表达有待进一步研究。

研究显示 CF 能分泌 IL-1β、IL-6 和 TNF-α 等细胞因子[26]。IL-1β 能减少胶原合成及提高 MMP (matrix metalloproteinase)活性，从而调节 CFs 的胶原代谢[27]。在成年大鼠 CFs，IL-1β 通过不同的机制增加 MMP2 和 MMP9 的表达与活性[28]。有报道 IL-1β 剂量依赖性地刺激大鼠 CFs 分泌整合素(fibronectin, FN)参与间质重塑[29]。IL-1β 比 TNF-α 更快地使 CFs 上调 AT1 的表达而参与心肌间质重塑[32,33]。本研究 RT-PCR 与芯片的结果都表明阻断 AT1 受体下调 IL-1β 的表达，阻断 AT2 受体上调 IL-1β 的表达。表明 AT2 受体在 IL-1β 的表达上与 AT1 受体起着不同的作用。

有研究报道 Ang Ⅱ和机械压力能诱导 CFs 分泌 TNF-α[30]。Sarkar 等报道 TNF-β 可直接作用于成纤维细胞，导致胶原纤维的合成分泌增多[31]。有研究表明 TNF-α 和 IL-1β 在 CFs 上调 AT1 受体的表达而参与心肌间质重塑[32,33]。压力超负荷下的左室心肌局部 TNF-α 表达异常增高，这可能与胶原增生、心室重构及心功能的损害有关；losartan 可能通过抑制 TNF-α 的过度表达从而减轻压力负荷下心室肌的胶原增生、心室重构及心功能的损害。本研究 RT-PCR 与芯片的结果都表明：阻断 AT1 受体下调 TNF-α 的表达，阻断 AT2 受体同样下调 TNF-α 的表达。该结果表明在调节 TNF-α 的表达上，AT2 与 AT1 受体起着相同的作用。

我们的研究表明 AT2 受体不仅在结构上、作用上与 AT1 受体不同，AT2 受体的信号转导通路与 AT1 受体比较明显不同。对单一途径的深入研究将是下一步工作的重点。IL-1β 和 TNF-α 是参与心肌间质重塑的两个重要细胞因子，本研究 RT-PCR 与芯片的结果都表明，AT2 受体参与两者的表达调控。

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