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Effects of Apelin on RAW264.7 cells under both normal and hypoxic conditions

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ABSTRACT

Macrophages are an important source of pro-inflammatory and pro-angiogenic factors, which can promote pathological processes involving inflammation and angiogenesis. This study investigated the effects of Apelin on macrophages under both normal and hypoxic conditions. Under normal culture conditions, Apelin down-regulated the mRNA expression levels of monocyte chemotactic protein 1 (MCP1), monocyte chemotactic protein 3 (MCP3), macrophage inflammatory protein 1 (MIP1α, MIP1β), vascular endothelial growth factor A (VEGFA), Angiopoietin 2 (Ang2) and tumor necrosis factor α (TNFα). The supernatant concentrations of MCP1, MCP3, MIP1α, MIP1β, macrophage inflammatory protein 2 (MIP2) and TNFα proteins were significantly decreased in the Apelin treated group. Hypoxia induced profound up-regulations of the angiogenic, chemokine, and inflammatory factors at both the mRNA and protein levels. Apelin suppressed the hypoxia-induced increases in MCP1, MCP3, MIP2, MIP1β and TNFα expression. The underlying mechanism of Apelin inhibit inflammation is regulating NF-κB/JNK signal pathway. Additionally, Apelin can protect macrophages from apoptosis and can enhance cell migration during hypoxia. And cleaved Caspase9/3 pathways were involved in Apelin inhibiting RAW264.7 apoptosis. In conclusion, we showed the effect of Apelin on RAW264.7 macrophage under normal and hypoxic condition, which could further influence the angiogenesis and inflammation process that promoted by macrophages.

Introduction

Angiogenesis is defined as the sprouting of new vessels from pre-existing ones [38]. Angiogenesis is a key pathologic process in many diseases, including cancer, diabetes, and other inflammatory conditions [19]. Additionally, pathological retinal angiogenesis is a major cause of vision loss in various diseases, such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration (AMD) [47,51]. Progression of physiologic angiogenesis is followed by a resolution phase marked by reduced endothelial cell proliferation and vessel stabilization [30]. Unlike physiologic angiogenesis, pathologic angiogenesis does not include a resolution phase and therefore results in a highly disorganized vascular network [30].

Hypoxia has long been recognized as a primary cause of pathologic angiogenesis [23]. However, the exact mechanisms underlying the induction of angiogenesis by hypoxia remain unclear and require additional study. In ischemic tissues, inflammatory cells that release cytokines, vasoactive molecules, and chemokines in response to the hypoxia are recruited; these cells then contribute to angiogenesis [6]. The resulting vascular networks are leaky, hemorrhagic and function poorly, leading to oxygen depletion and extracellular acidosis [9,31]. Thus, a vicious cycle of hypoxia and pathologic angiogenesis is established.

As early as the late 1970s, researchers began to recognize that macrophages are the key to angiogenesis [34]. Recent evidence has suggested that macrophages play major roles in the regulation of angiogenesis in both normal and diseased tissues [24]. When stimulated by hypoxia, macrophages adapt to unfavorable conditions [5] and secrete an array of angiogenic cytokines, growth factors [45], inflammatory factors, chemokines and proteolytic enzymes [43]. These molecules include VEGF (vascular endothelial growth factor) [15,22,25], TNF-α (tumor necrosis factor α) [1,2,8],

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IL-6 (interleukin-6) [27,29], MMPs (matrix metalloproteinases) [35,37,53], CXC chemokines and CC chemokines [41]. Chemokines have disparate effects on angiogenesis regulation [33], as several members of the CXC and CC chemokine families are potent inducers of angiogenesis, while a different subset of CXC chemokines are angiostatic [41].

In previous work from our lab, we focused on investigating the role of Apelin/APJ. The Apelin peptide, an endogenous ligand for the angio-tensin-1-like receptor APJ [46]. The pre-apelin contains 77 amino acids. It can be cleaved into several biologically active forms of Apelin peptides in vivo, such as Apelin-13 (Apelin 65–77) [46], the most bioactive one. We found that Apelin supports primary rat retinal Müller cells under conditions of chemical hypoxia and glucose deprivation [48]. Apelin-13 induces proliferation, migration, and collagen I mRNA expression in human retinal pigment epithelium (RPE) cells via the PI3K/Akt and MEK/Erk signaling pathways [36]. Recent research has demonstrated that Apelin down-regulates MCP-1 and TNFα expression by macrophages, and reduces the infiltration of macrophages around lesions [26].

However, the influence of Apelin on macrophages remains unclear. To investigate the relationship between macrophages, Apelin, and angiogenesis, we studied the effects of Apelin on RAW264.7 cells in both normal and hypoxic conditions.

Materials and methods

Cell culture

RAW264.7 cells were purchased from ATCC (TIB-71™) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, 11965-092) containing 10% fetal bovine serum (FBS; Gibco, 10099-141). These cells are from a macrophage line transformed with Abelson murine leukemia virus. For subcultivation, the cells were cultured at 37 °C with 5% CO_2, and the medium was replaced every 2 days. For biological replicates, we used different batches of cryopreserved cells, avoided continued subcultivation change the character of cells.

For hypoxia induction, cells were maintained in a sealed chamber (C-Chamber Manual version 0.2 d0410, BioSpherix, Ltd., Lacona, NY, USA) that was flushed with a humidified gas mixture composed of 1% oxygen (O_2), 5% carbon dioxide (CO_2) and 94% nitrogen (N_2). The oxygen partial pressure value (pO_2) in the chamber was controlled and validated using a Compact Oxygen Controller (Proox Model C21 Manual version 0.2 d0506, BioSpherix, Ltd., Lacona, NY, USA). Under hypoxia control mode, oxygen probe and carbon dioxide probe detect the gas concentration in the C-Chamber continuously and transfer the gas concentration value to the controller Proox Model C21. When the gas concentration changes, the Proox Model C21 control the high purity nitrogen and carbon dioxide filling into the C-Chamber to reduce oxygen concentrations. The hypoxic cell culture chamber was kept closed throughout the entire experimental procedure.

Synthetic Apelin-13 peptide was purchased from Sigma (St. Louis, MO, A6469).

The amino acid Sequence of Apelin-13 peptide: Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe.

We used DMEM culture medium to dilute the Apelin-13 trifluoroacetate salt powder into 1 mg/ml Apelin-13 solution, and aliquoted the stock solution, then stored −80 °C. During the experiments, we used 1% FBS DMEM to dilute the 1 mg/ml Apelin-13 solution into the required concentration. Cells were incubated with varying concentrations of Apelin (1000 ng/ml, 500 ng/ml and 100 ng/ml) for the respective assays. All of the assays below performed at least three times biological replicate.

Real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from RAW264.7 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The final RNA were eluted in 10 μl distilled diethylpyrocarbonate-treated water. The purity and concentration of RNA were measured using the Gene Quant pro device (Nanovue Spectrophotometer, GE Healthcare, London, UK). The A260/A280 ratio of optical density was between 1.9 and 2.0 for all RNA samples, indicating sufficient quality. cDNA was synthesized from 5 μg total RNA using a RevertAid First Strand cDNA Synthesis Kit (ThermoScientific, #k1622). The mRNA expression levels were determined with a real-time PCR detector (ThermoFisher Scientific PikoReal 96,5100). The PCR solution contained 300 ng of cDNA, 0.2 μM specific primers each, 5 μl Maxima SYBR Green qPCR Master Mix (ThermoScientific, #k0221), and water with a final volume of 10 μl. The PCR primer pair sequences used for each gene are shown in Table 1. The reaction conditions for amplifying DNA were 95 °C for 10 min, followed by 37 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s. mRNA expression was normalized to β-actin, Gapdh and B2m expression. mRNA expression was calculated using the following equation: Fold change = $2^{-\Delta\Delta CT}$.

Western blot analysis

Expression levels of the APJ receptor and cell signal pathways in RAW264.7 cells in normoxic and hypoxic environments were analyzed by Western blotting. Two 25 ml culture bottle planted with 5×10^6 cells respectively were prepared for the Western blot assay. After replacing the medium with 1% FBS DMEM, cells were cultured in hypoxic and normoxic conditions respectively. After 24 h incubation, protein was extracted for Western blotting. The RAW264.7 cells were extracted with Procarta Cell Lysis Buffer (Cat.NO.EPX-99999-000). After centrifugation, the supernatant was collected, and the protein lysate was measured with a BCA protein assay kit (Novagen, Cat 71285-3) according to the manufacturer's instructions. Protein samples were run on 15% SDS–PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes

Table 1
Mouse primers used for real-time PCR.

Gene name		Sequence 5′–3′
Ccl7 (MCP-3)	Forward primer	GCTGCTTTCAGCATCCAAGT
	Reverse primer	CGACTACTGGTGATCCTTCTG
Ccl2 (MCP-1)	Forward primer	GTGTCCCAAAGAAGCTGTAGT
	Reverse primer	TGTATGTCTGGACCCATTCC
Ccl3 (MIP-1α)	Forward primer	ATTCCACGCCAATTCATCGT
	Reverse primer	CAGGTCTCTTTGGAGTCAGC
Ccl4 (MIP-1 β)	Forward primer	TATGAGACCAGCAGTCTTTGC
	Reverse primer	TCAACTCCAAGTCACTCATGT
Cxcl2 (MIP-2)	Forward primer	AGACAGAAGTCATAGCCACTCTC
	Reverse primer	GCTCCTCCTTTCCAGGTCAGTT
Csf1 (M-CSF)	Forward primer	TTACCAAGAACTGCAACAACAG
	Reverse primer	GGGTGGCTTTAGGGTACAGG
Tnf (TNF-α)	Forward primer	ACCACGCTCTTCTGTCTACTG
	Reverse primer	AGAAGATGATCTGAGTGTGAGG
Vegfa (VEGFA)	Forward primer	TGTGGACTTGTGTTGGGAGGAGGA
	Reverse primer	GTGCCCTGGCCTTGCTTGCT
Angpt2 (Angiopoietin 2)	Forward primer	GCTCCAGGTGCTGGTGTCCAA
	Reverse primer	TCAGCAAGCTGTTGACGGTCTCC
Aplnr (APJ)	Forward primer	TCCGACGGCTGGTCCGTATCT
	Reverse primer	GGCCCAGCTCCAGACTTCTCCA
B2m	Forward primer	CCACCCACCGGAGAATGGGA
	Reverse primer	CCCGTTCTTCAGCATTTGGATTTCA
Actb	Forward primer	CCACCATGTACCCAGGCATTGCT
	Reverse primer	GCCAGGATGGAGCCACCGAT
Gapdh	Forward primer	GGCGCGCGTCATCAGCTC
	Reverse primer	TGCGGCCGTCTCTGGAACAG