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G-3. 新聞報道

なし

H. 知的所有権の取得状況

なし

厚生労働省科学研究補助金
（医療技術実用化総合研究事業）
平成２１年度分担研究報告書

バイオニック血圧制御システムの実用化研究
瞬時血圧測定法の開発（分担課題名）

分担研究者　山越　憲一（金沢大学 理工研究域　教授）

研究要旨：
脊髄損傷患者の自律神経障害に起因する起立性低血圧症を予防するためのバイオニック血圧制御システムの実用化研究として，今年度は足背動脈を対象とした非侵襲瞬時血圧計測システムの設計開発を行った。実用化のための重点開発項目として，①各被測定者の足型に合わせたカフ・光電センサ部を含む固定具の開発、および②瞬時血圧計測のための制御方式として Self-tuning 適応制御系を実装したシステムの試作開発を行った。その結果，健常者を対象とした性能試験において，非侵襲瞬時血圧計測システムの有効性を確認した。

A. 研究目的

現在，世界における脊髄損傷患者(以下脊損者)は毎年約 13 万人以上増加しており，累計 120 万人以上いるとされている。このうち日本においては 10 万人以上の脊損者がいるとされている。脊損者の多くは自律神経障害などの合併症を発症し，特に脊損者の 70%以上が起立性低血圧症による QOL の低下に悩まされている。

本分担研究では，脊損者の QOL 向上のためにバイオニック血圧制御システムの実用化研究として，非侵襲瞬時血圧計測・制御システムの開発を進めている。今年度は足背動脈を対象とした瞬時血圧計測システムの設計・試作開発を行うことを目的とした。

B. 研究方法

B-1. 非侵襲瞬時血圧計測システムの開発に関する研究

図 B-1 にバイオニック血圧制御システムの全体概要ブロック図を示す。本システムでは，脊損者の瞬時血圧を計測し，目標血圧との差分から血圧制御装置により脊損者の血圧を制御することで、上体起こしや起立などの姿勢変化支援を行うものである。平成 21 年度は，分担研究者が提案した容積補償法に基づき，脊損者の負担にならない足背動脈を対象とした非侵襲瞬時血圧計測プロトタイプシステムの設計・試作開発，ならびに健常者における評価を行った（図 B-2）。

実用化のための重点開発項目としては，容積補償法による瞬時血圧計測を実施するにあたり，光電センサによる対象血管の容積脈波信号の検出，および対象血管内容積を一定とするカフ圧の制御を行う必要があることから，①体動などによるカフ・光電センサ部のズレを防ぐために被測定者の足型に合わせた 3 次元 CAD による固定具の開発（造型機（uPrint，丸紅情報システムズ）を使用），②対象血管やその周辺組織の特性が変化しても適切な計測・制御を行うための Self-tuning 適応制御系(オンラインシステム同定，極配置によるフィードバックゲインの決定）の開発（DSP コントローラ（MABX 1501/815，dSPACE Japan 株式会社）を使用）を行い，健常者（n=3，20-23yrs）における姿勢変化時の瞬時血圧変化を評価した。なお，計測した瞬時血圧値の評価には直接法が望ましいが，今回は参考値として市販の上腕血圧計を用いて 2 分毎に比較した。

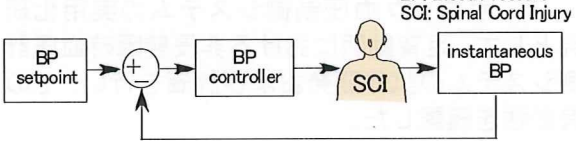

図 B-1　バイオニック血圧制御システム

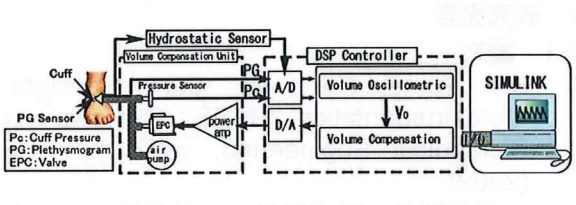

図 B-2　非侵襲瞬時血圧計測システム

C. 研究結果

C-1. 非侵襲瞬時血圧計測システムの開発に関する研究

図 C-1 に本実験により得られた瞬時血圧のグラフ（水頭圧差補正済）と市販血圧計の値をプロットしたものを示す。図 C-1 から，臥位から長座位への姿勢変化の際に血圧の降下 - 回復反応が確認された。また，市販血圧計とも同様の値を取ることが確認された。

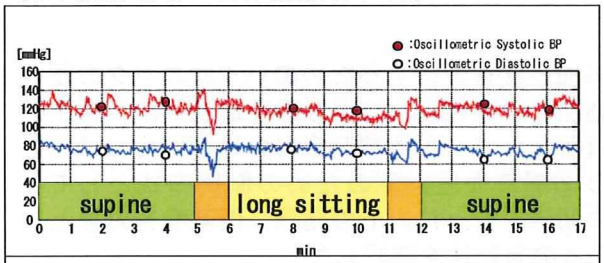

図 C-1　非侵襲瞬時血圧計測システム計測例

D. 考察

バイオニック血圧制御システムの実用化研究として，非侵襲瞬時血圧計測システムの試作開発および評価を行った。実用化の際に問題となると予想されるカフ・光電センサ部の固定方法や制御方式については，被測定者に合わせた固定具の作成、ならびに適応制御系の導入により解決を図った。今後，長期計測時の本計測システムの妥当性・安定性について検討を重ねる必要がある。また，実際の脊損者において，瞬時血圧計測システムを組み込んだバイオニック血圧制御システムの開発・評価については，平成 22 年度以降，順次進める予定である。

E. 結論

バイオニック血圧制御システムの実用化研究として，足背動脈における非侵襲瞬時血圧計測システムの試作開発および評価を行い，その有効性を確認した。

F. 健康危険情報

特になし

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G-3. 新聞報道

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H. 知的所有権の取得状況

なし

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Original article

Differential regulation of TNF receptors by vagal nerve stimulation protects heart against acute ischemic injury

Rajesh G. Katare [a,1], Motonori Ando [a,c], Yoshihiko Kakinuma [a], Mikihiko Arikawa [a], Fumiyasu Yamasaki [b], Takayuki Sato [a,*]

[a] Department of Cardiovascular Control, Kochi Medical School, Nankoku, Japan
[b] Department of Clinical Laboratory, Kochi Medical School, Nankoku, Japan
[c] Department of Science and Education, Okayama University, Okayama, Japan

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ABSTRACT

Vagal nerve stimulation (VS) has been reported to improve the survival after both acute and chronic myocardial infarction through the release of neurotransmitter ACh. However, the precise mechanism behind its beneficial effect is still unknown. In this study, we demonstrate the upregulation of tumor necrosis factor-alpha (TNF-α) and its cell survival TNF receptor-2 (TNFR2) as the mechanism behind VS induced myocardial protection. We investigated the effects of efferent VS on myocardial ischemic injury with *in vivo* and *in vitro* mouse models. In *in vivo* hearts VS significantly increased the expression of TNF-α both at the messenger and protein level after 3-hours of myocardial ischemia. In the *in vitro* studies ACh treatment before hypoxia, induced a significant upregulation of TNF-α compared to the untreated cardiomyocytes. Immunofluorescence analysis confirmed the synthesis of TNF-α by cardiomyocytes both in vivo and in vitro. VS also significantly reduced the myocardial infarct size ($23.9 \pm 5.7\%$ *vs.* $56 \pm 1.9\%$) and activated the cell survival Akt cascade system. Further, ACh upregulated the cell survival TNFR2 expression, while downregulating the cell destructive TNF receptor 1 (TNFR1) expression. These results were confirmed using the TNF receptors deficient mice, where the VS mediated protection was lost both in vivo and in vitro in TNFR2 ($TNFR2^{-/-}$) and TNF receptors double knock out ($TNFR1^{-/-}2^{-/-}$) mice. VS and ACh protects the heart against acute ischemia or hypoxic injury by differentially regulating the TNF receptor subtypes.

1. Introduction

Acute myocardial ischemia is one of the leading cause for sudden death [1]. Despite of the various researches to find out a new strategy in preventing the acute ischemia induced myocardial damage, the treatment and prognosis remains limited. Hence any advancement for the treatment of acute ischemia would be highly beneficial in improving the quality of life in patients with acute myocardial infarction.

Vagal nerve stimulation (VS) has been demonstrated to improve the survival in animal model of chronic heart failure [2]. Our previous study using rats [3] demonstrated that efferent VS, before or during 30 min of left coronary artery (LCA) occlusion, protected hearts against ischemia-induced lethal arrhythmias by prevention of the loss of functional gap-junction channels. In another study we demonstrated the protective effect of VS was mediated through the release of ACh at terminal nerve endings [4]. Although these studies have clearly enlightened the protective role of VS against myocardial stress, the exact mechanisms through which this stimulus exhibits its effects still remains unknown. Moreover, VS is being currently used clinically in treating the patients with heart failure [5] and hence it becomes extremely necessary to understand the mechanism by which VS exerts its cardio protective effect. An elegant study by Borovikova et al, demonstrated the inhibition of tumor necrosis factor-α (TNF-α) in the liver as one possible mechanism behind the anti inflammatory effect afforded by efferent VS [6]. Tumor necrosis factor-α is a proinflammatory cytokine with pleiotropic biological effects that has been demonstrated to produce either adaptive homeostatic responses or devastating maladaptive effects, depending on the duration and degree to which this cytokine is expressed [7,8]. Interestingly, our preliminary studies showed a marked increase in the TNF-α mRNA with VS treatment after 30 min of acute ischemia (supplemental Fig. 1). Detailed search for the earlier studies with TNF-α showed that, the effect of TNF-α on heart was dependent on the type of receptor with which it binds [9,10]. There are 2 distinct cell

* Corresponding author. Department of Cardiovascular Control, Kochi Medical School, Nankoku, Kochi 783-8505, Japan. Tel.: +81 88 880 2309; fax: +81 88 880 2310.
E-mail address: tacsato-kochimed@umin.ac.jp (T. Sato).

[1] The author has now moved to Bristol Heart Institute, University of Bristol, Bristol, UK.

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surface receptors of TNF family, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Higuchi et al. [11] demonstrated the differential regulation of cell survival, cardiac dysfunction and remodeling by TNFR1 and TNFR2 receptors. Thus the role and mechanism of TNF-α on heart remains highly controversial.

With these factors in mind, we hypothesized that VS during ischemia would exert its protective effect by upregulation of TNFR2, the cell protective receptor of TNF family and prevent acute ischemia induced myocardial damage. Therefore in the present study using both *in vivo* model of acute myocardial ischemia and *in vitro* model of primary cultured cardiomyocytes and TNF receptor deficient cardiomyocytes, we studied the effect of VS and acetylcholine (ACh) treatment on upregulation of TNF receptors, and their pathway in mediating protection against acute ischemic or hypoxic insult to the myocardium.

2. Methods

Male C57BL6 mice (SLC, Japan), aged between 8 and 10 weeks and weighing between 30 ± 2 grams, TNF receptors deficient mice (TNF receptor 2 deficient ($TNFR2^{-/-}$), TNF receptor 1 deficient ($TNFR1^{-/-}$) and TNF receptors double knock out ($TNFR1^{-/-}2^{-/-}$)) weighing between 30 ± 2 grams and 2-day-old neonates of C57BL6 and TNF receptor deficient mice (Jackson Laboratories, USA) were used. Wild type age matched litter mates of TNF receptor deficient mice were used as control animals. Details regarding the development of the TNF receptor deficient strain is available at http://www.jax.org/. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication No. 86–23, revised 1985).

2.1. Experimental Models

This study was designed to clarify exact mechanisms behind the protective effects of VS during acute ischemia, which was studied using three different protocols.

Protocol 1: Demonstration of the protective effect of VS against acute myocardial ischemia using C57BL6 mice.
Protocol 2: Understanding the exact mechanisms in VS induced protection and time course in the regulation of TNF-α and its receptors using in vitro model of primary cultured cardiomyocytes from C57BL6 mice treated with ACh, a neurotransmitter released by VS at cardiac nerve endings. We previously demonstrated that VS induced protection is mainly mediated through the release of ACh [4].
Protocol 3: Confirmation of TNFR2 receptor upregulation by VS using TNF receptors deficient mice ($TNFR1^{-/-}$ and TNFR2.

2.1.1. Acute Myocardial Ischemia and Hypoxia Models

2.1.1.1. In Vivo Model and Vagal Nerve Stimulation. Preliminary studies with 30 min of left coronary artery (LCA) ligation demonstrated significant upregulation in the messenger level of TNF-α by VS (Supplemental Fig. 1). However with in this short time interval we did not observe any difference in the protein level of TNF-α in the myocardium. Therefore, we used 3 h LCA ligation model (MI-SS group, n = 13) which would allow ample time to study the protein expressional changes within the myocardium. Vagal nerve stimulation with MI was performed as described in our earlier studies [3,4,12]. In brief right vagal nerve was stimulated with continuous electrical rectangular pulses of 0.1-ms duration at 10 Hz throughout LCA ligation. Detailed description can be found in the online supplemental methods section.

2.1.2. In Vitro Cellular Model and ACh Treatment

2.1.1.2.1. Primary Cultured Cardiomyocytes and ACh treatment. Cardiomyocytes were isolated from 2-day-old neonatal C57BL6, TNF receptors deficient and their wild type littermate mice and cultured, as described previously [3]. After 4 days of culture, the serum starved cells were treated with ACh (0.5 mM) for 15 min, followed by exposure to hypoxia. Our earlier studies demonstrated ACh release as the major mechanism behind VS induced protection, as inhibition with Atropine completely blocked the effect of VS in vivo and ACh in vitro [3,4] **(referene)**. Detailed description can be found in the online supplemental methods section.

2.2. Infarct Size Assessment and MTT Assay of Cell Viability

In the *in vivo* model study, the infarcted area was determined by TTC staining as described previously [13]. The infarcted area was measured with Image-Pro version 4.0 (Media Cybernetics).

To evaluate the effect of ACh on viability of hypoxic cardiomyocytes, we employed a colorimetric method with an MTT Cell Count Kit (Nacalai Tesque) as described earlier [14].

2.3. Quantitative RT PCR

Total RNA was isolated from LV samples (Trizol, Invitrogen, UK) and reverse transcribed (Sensiscript reverse transcriptase, Qiagen). Quantitative PCR (qPCR) was performed in a LightCycler (Roche, Burgess Hill, UK) using Platinum taq polymerase (Qiagen) and the primer pairs listed below. For quantification, mRNA amount of the respective gene was normalized to the amount of18S rRNA using the 2—DDCT method. Each reaction was performed in triplicate.

18S rRNA	forward: 5′- TAGAGGGACAAGTGGCGTTC -3′ reverse: 5′- TGTACAAAGGGCAGGGACTT -3′
TNF-α	forward: 5′- CCGATGGGTTGTACCTTGTC -3′ reverse: 5′- GTGGGTGAGGAGCACGTAGT -3′
TNFR1	forward: 5′- GACCGGGAGAAGAGGGATAG -3′ reverse: 5′- GTTCCTTTGTGGCACTTGGT -3′
TNFR2	forward: 5′- CTTACCCCAGCCAGTGTCC -3′ reverse: 5′- AAGGAGGTGCTTGGAGCAG -3′

2.4. Protein Preparation and Immunoblotting

Protein samples extracted from left ventricle were processed for immunoblotting as described earlier [3,4,13]. Detailed description can be found in the online supplemental methods section.

2.5. Myocardial TNF-α Levels

Myocardial TNF-α in LV tissue and cardiomyocytes culture supernatant was determined using a commercially available ELISA set (R&D Systems Inc, Minneapolis, Minn). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in triplicate.

2.6. Immunocytochemistry and Confocal Microscopy

At the end of different time intervals of hypoxia, cells were fixed with 4% freshly prepared formaldehyde and then immunostained according to the standard protocols. After washing with PBS and blocking for 1 h, cells were incubated overnight with primary antibodies against TNF-α (diluted 1:100, Santacruz), phospho Akt (diluted 1:100, Cell Signaling) and NFκ-B (diluted 1:1000, Santacruz) and thereafter incubated for 4 h in Alexa488 or Alexa546-conjugated goat anti-rabbit IgG (phospho Akt and NFκ-B) or rabbit anti-goat IgG (TNF-α) (Molecular Probes) diluted 1:100. Fluorescence of Alexa488

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and Alexa 546 was observed using a confocal laser scanning microscope system (FV300, Olympus).

2.7. Caspase-3 Activity Assay for Detection of Apoptosis

To assess the effect of ACh on hypoxia induced apoptosis, caspase-3 activity was measured using a Caspase-3/CPP32 Colorimetric Assay Kit (BioVision) [4]. The myocardial sample or cultured cells were lysed and caspase-3 substrate was added to the lysate. Caspase activity was measured with a spectrophotometer (Ultrospec 3000, Pharmacia) according to the manufacturer instructions.

2.8. Statistical Analysis

When normality test fails, nonparametric multiple-comparison tests among three or more groups were performed by a Steel-Dwass

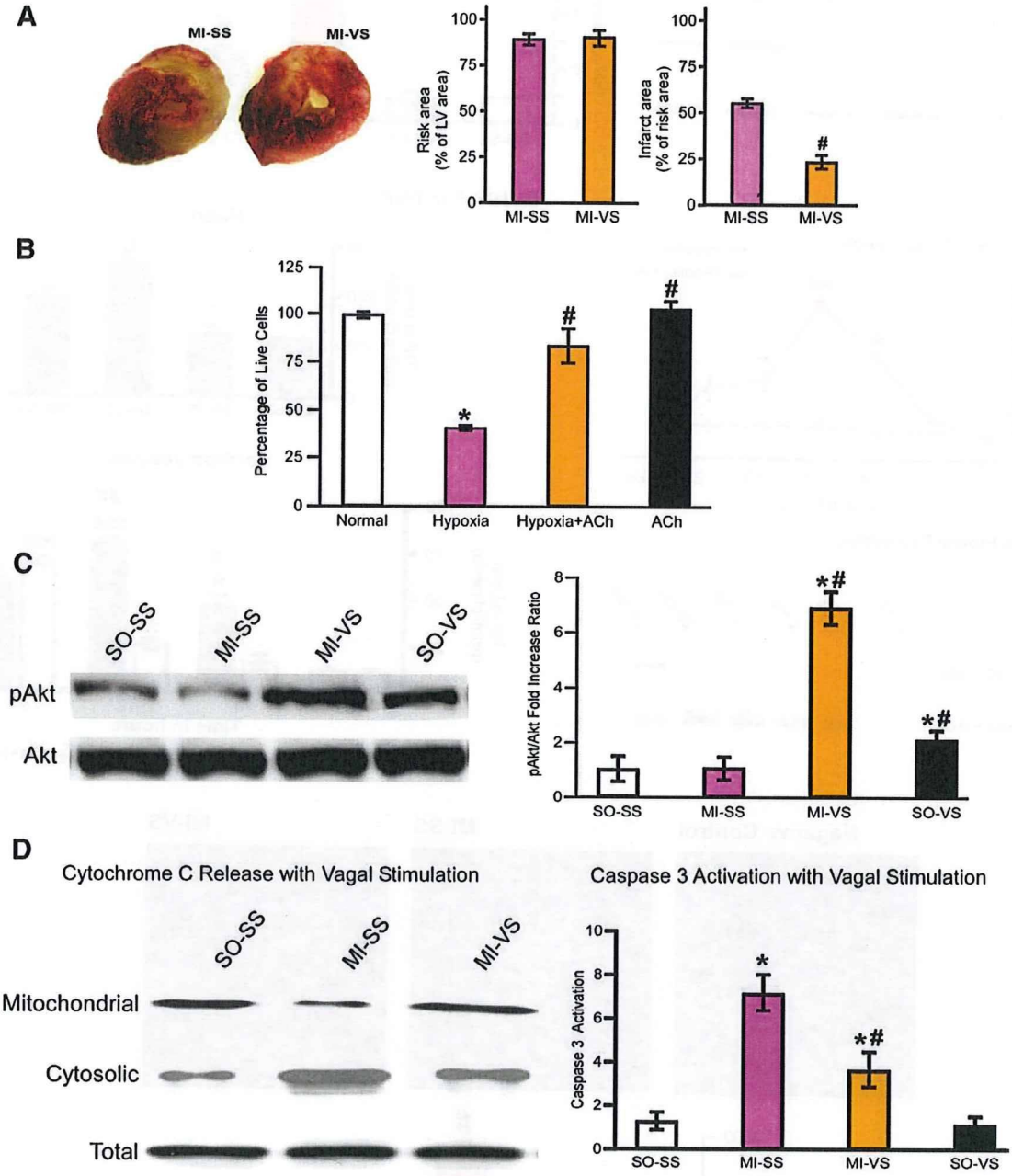

Fig. 1. Effect of VS or ACh after Myocardial Ischemia. (**A**) Effect of VS on infarct size after acute ischemia. Representative TTC staining and quantitative analysis showing the percentage of infarct area after 3 h of myocardial ischemia. n = 8 for each group Values are expressed as mean±SD. #$P<0.001$ vs MI-SS. (**B**) Bar Graph showing the percentage of viable cells after hypoxia with or without ACh treatment. For each treatment group, 5 culture dishes were analyzed. Values are expressed as mean±SD. *$P<0.001$ from Normal; # $P<0.001$ vs Hypoxia. (**C**) Expression of cell survival Akt with vagal stimulation. ***Left Panel*** – Representative immunoblot showing the expression of pAkt and Akt between the study groups. ***Right Panel*** – Bar graph showing the densitometry analysis of ratio between pAkt and Akt expression after myocardial ischemia. Data represented as the fold change compared to the SO-SS group. n = 5 for each group. Values are expressed as mean±SD. *$P<0.01$ vs SO-SS, # $P<0.01$ vs MI-SS. (**D**) Inhibition of proapoptotic signals by VS. ***Left Panel*** – Representative immunoblot showing the expression of cytochrome C in mitochondrial and cytosolic fractions. VS significantly inhibited the cytosolic release of cytochrome C. ***Right Panel*** – Bar graph demonstrating the inhibition of caspase-3 activation by VS during myocardial ischemia. Data represented as fold increase in caspase-3 activation compared to the SO-SS group. n = 5 for each group. Values are expressed as mean±SD. *$P<0.01$ vs SO-SS, # $P<0.01$ vs MI-SS. SO-SS – sham operation sham stimulation; MI-SS – myocardial ischemia sham stimulation; MI-VS – myocardial ischemia vagal stimulation; SO-VS – sham operation vagal stimulation.

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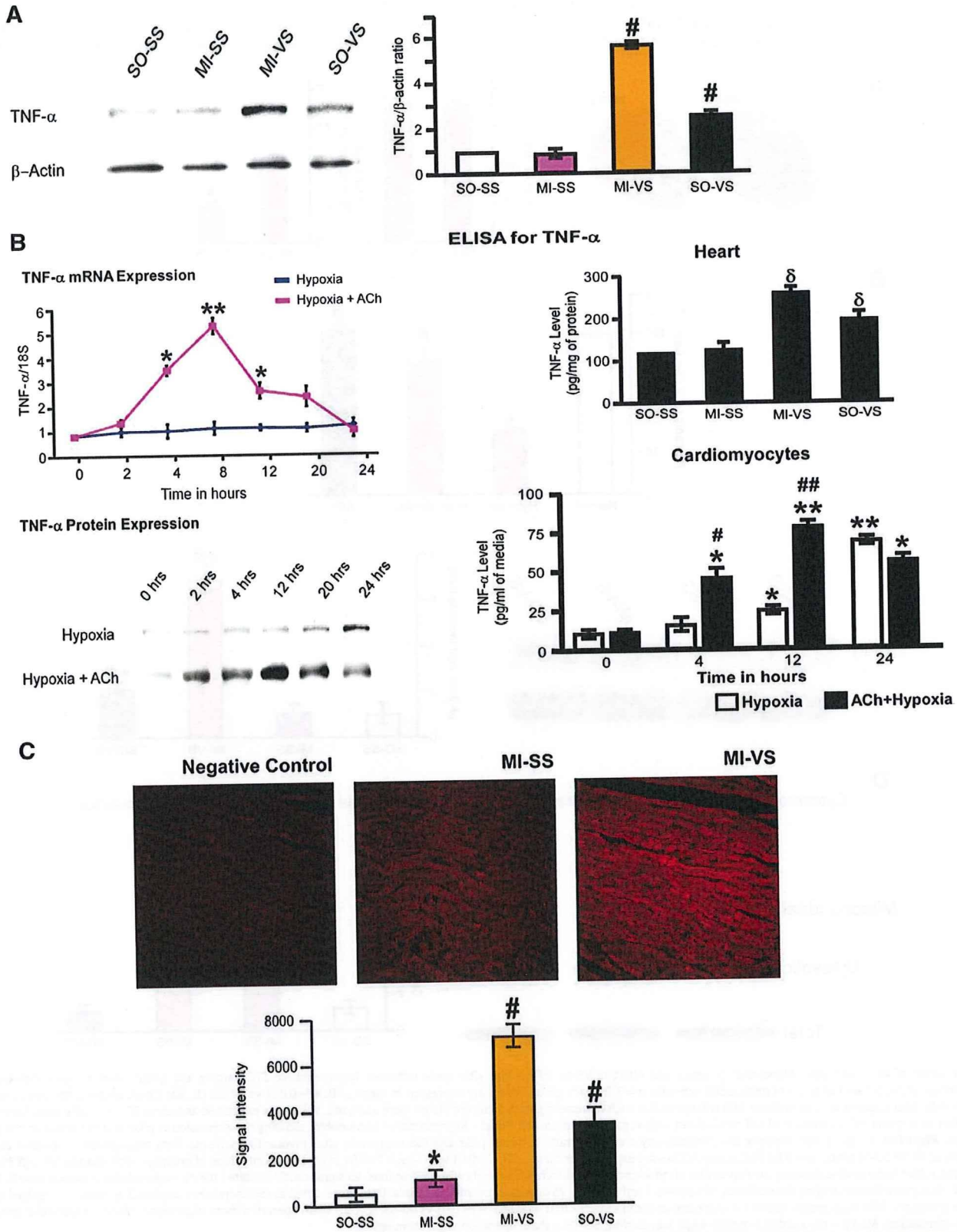

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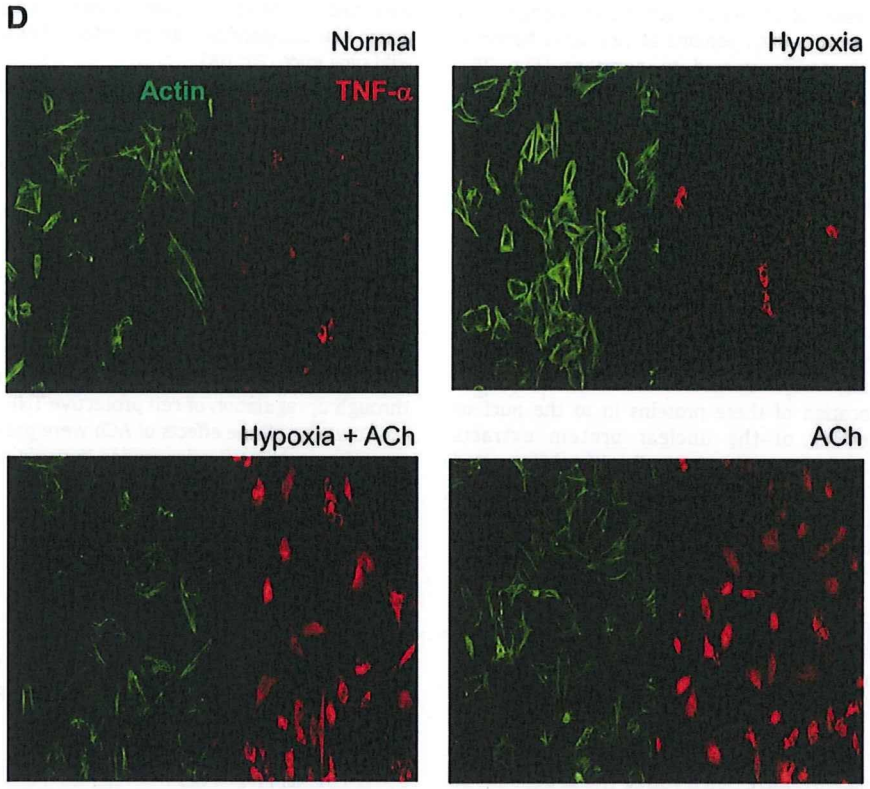

Fig. 2. Effect of VS and ACh on Activation of TNF-α. (**A**) Activation of TNF-α by vagal stimulation. ***Left Panel*** - Representative immunoblot picture showing the expression changes in TNF-α among the study groups. ***Right Panel*** – Bar graph demonstrating the densitometric analysis of TNF-α in the study groups. VS significantly increased the expression of TNF-α after myocardial ischemia. Values are normalized by reference levels of beta actin expression and expressed as mean±SD. n = 5 for each group. #*P*<0.05 vs MI-SS. (**B**) Activation of TNF-α expression by ACh treatment. **Left Panel:** Quantitative real-time PCR (***above***) and representative immunoblot (***below***) picture (n = 5 in each group) showing the expression of TNF-α in primary cultured cardiomyocytes subjected to hypoxia with or without ACh treatment. ACh significantly increased the expression of both messenger and protein levels of TNF-α. The increase could be observed starting from 4 h of hypoxia and the peak is reached at 12 h. n = 5 for each group. Data expressed as mean±SD. **P*<0.01 and ***P*<0.001 vs hypoxia at corresponding time point. **Right Panel:** Bar graphs showing the amount of TNF-α in heart (n = 5) and cardiomyocytes (n = 4) by ELISA. Data expressed as mean±SD. δP<0.05 vs SO-SS; *P<0.05 and **P<0.005 versus 0 h of corresponding treatment; #P<0.05 and ##P<0.005 vs hypoxia at corresponding time point. (**C**) Representative confocal microscopic pictures (***upper panel***) and quantitative analysis of the fluorescence intensity (***lower panel***) showing the TNF-α expression (red color) in the myocardium by cardiomyocytes 3 after myocardial ischemia. Values represent the fluorescence intensity and expressed as mean±SD. n = 5 for each group. **P*<0.01 vs SO-SS, #*P*<0.05 vs MI-SS. (**D**) Synthesis of TNF-α in cultured cardiomyocytes. Representative confocal microscopic pictures showing TNF-α expression in cultured cardiomyocytes after hypoxia. Actin fibers are stained in green and TNF-α in red. Hypoxia alone does not have any change on TNF-α expression, whereas, addition of ACh significantly increased the TNF-α expression. ACh in the absence of hypoxia also increased the synthesis of TNF-α in the cardiomyocytes. SO-SS – sham operation sham stimulation; MI-SS – myocardial ischemia sham stimulation; MI-VS – myocardial ischemia vagal stimulation; SO-VS – sham operation vagal stimulation.

testcxx [15,16] with Excel Statistics version 5.0 (Esumi). Differences were considered to be statistically significant at a level of overall $P<0.05$. Values are expressed as mean±SD.

3. Results

3.1. Effects of VS and ACh on Ischemic and Hypoxic Injury

3.1.1. VS Reduced Myocardial Infarct Size

Myocardial infarct size is the direct evidence for the degree of myocardial damage in the form of necrosis. VS induced a marked reduction in the area of infarct size with MI-VS group as compared with the MI-SS group (23.94±5.7% in MI-VS *versus.* 56±1.9% in MI-SS, $P<0.001$), Fig. 1A).

3.1.2. ACh treatment Improved Cell Viability

Similar to the in vivo studies ACh treated cardiomyocytes exhibited an improved viability when compared to untreated cells after 12 h of hypoxia (81±3.2% *versus* 40.4±3.5%, $P<0.001$, Fig. 1B).

3.1.3. VS and ACh Activated Cell Survival Signaling Cascades

VS significantly induced activation of Akt in the form of increased phosphorylation compared sham stimulation (6.98±0.6 *vs* 1.1±0.06, $P<0.001$) (Fig. 1C). Even in the absence of myocardial ischemia, VS activated the phosphorylation of Akt (2.15±0.3 fold higher, $P<0.001$ vs SO-SS). Same results were observed on treating the cultured cardiomyocytes with ACh after 12 h of hypoxia (Supplementary Fig. 2).

3.1.4. VS Reduced Activation of Proapoptoic Signaling Cascade

VS inhibited MI induced apoptotic cell death by inhibiting the cytochrome C release and caspase 3 activation (3.12±0.4 *versus* 7.26±0.4, fold increase compared to SO-SS, $P<0.001$) (Fig. 1D). ACh demonstrated similar effects on cardiomyocytes after hypoxia (Supplementary Fig. 3).

3.2. Effect of VS and ACh on TNF-α and its Receptors

3.2.1. In vivo VS Upregulated TNF-α Expression

VS significantly upregulated TNF-α protein after 3 h of MI (5.71±0.08 *vs* 0.38±0.01, $P<0.001$, by densitometry analysis) (Fig. 2A), which was also detected in the SO-VS group (2.41±0.09 fold increase compared with SO-SS) suggesting the VS specific effect on TNF-α.

3.2.2. In vitro ACh Treatment Induced Cardiac Synthesis of TNF-α

The source for TNF-α following VS was from the cardiomyocytes. Treating the primary cultured cardiomyocytes with ACh showed a

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time dependent upregulation of TNF-α at both the messenger and protein level with a the protein level peaking at 12 h after hypoxia, following which the expression started to decrease (Fig. 2B). Measurement of TNF-α protein by ELISA confirmed our findings (Fig. 2B). In contrast, the vehicle-treated group showed an increase in the TNF-α expression from 20 h of hypoxia. Immunostaining confirmed the synthesis of TNF-α by the cardiomyocytes in response to VS (Fig. 2C) or ACh treatment (Fig. 2D).

3.2.3. Nuclear Factor - Kappa B Induce the Synthesis of TNF-α in Cardiomyocytes

The mechanism of TNF-α synthesis in the cardiomyocytes by VS was through activation of Nuclear Factor - Kappa B (NFκ-B). Immunocytochemistry showed the localization of NFκ-B in cytoplasm of hypoxic cardiomyocytes, where as ACh treatment with or without hypoxia induced translocation of these proteins in to the nucleus (Fig. 3A). Immunoblotting of the nuclear protein extracts demonstrated the increased expression of NFκ-B in the ACh treated group starting from 2 h of hypoxia ($P<0.05$ *vs* untreated group, Fig. 3B). This was further confirmed by increased phosphorylation of IκB-α in the ACh treated group as early as 2 h of hypoxia ($P<0.05$ *versus* untreated group, Fig. 3C).

To further confirm if NFκ-B regulates the ACh induced synthesis of TNF-α in cardiomyocytes, we inhibited the activity of NFκ-B using a potent inhibitor pyrrolidine dithiocarbamate (PDTC). Treating the cells with PDTC before ACh, markedly inhibited ACh induced upregulation of TNF-α ($P<0.05$) at 12 h of hypoxia, (Fig. 3D).

3.2.4. Differential Regulation of TNF Receptors by VS

The consequences following increased levels of TNF-α depends on the receptor subtypes with which it binds. ACh treatment during hypoxia induced the upregulation of cell protective TNFR2 receptor in time dependent manner starting from 2 hours after hypoxia (Fig. 4A). In contrast, the expression of cell destructive TNFR1 was upregulated in the vehicle-treated cardiomyocytes, while ACh treatment inhibited this upregulation (Fig. 4B). Finally, the ratio between TNFR2 and TNFR1 confirms that, the TNF-α synthesized in response to VS binds with the cell protective TNFR2 and protects cardiomyocytes against hypoxic injury (Fig. 4C).

3.3. Effect of VS on TNF Receptor Deficient Mice

3.3.1. Loss of Protective Effect on Infarct Size

To clarify the TNFR2 mediated cardio protective pathway by VS, we studied the effect of VS on acute MI using the $TNFR1^{-/-}$, $TNFR2^{-/-}$ and $TNFR1^{-/-}$ $R2^{-/-}$ mice. VS reduced the infarct size in $TNFR1^{-/-}$ mice (23.4 ± 4.4 *vs* 44.19 ± 3.5, $P<0.001$) where as this protective effect was lost in the $TNFR2^{-/-}$ (38.2 ± 5.2 *vs* 44.93 ± 4.7, $P=0.06$) and $TNFR1^{-/-}2^{-/-}$ (43.69 ± 1.9 *vs* 47.66 ± 4.5, $P=0.066$) mice confirming the role for TNFR2 in VS induced cardioprotection (Fig. 5A).

3.3.2. Reduced Activation of Cell Survival signaling

The marked increase in the activation of cell survival Akt observed with VS was inhibited in the $TNFR2^{-/-}$ mice (1.39 ± 0.03 *vs* 0.84 ± 0.06) and $TNFR1^{-/-}2^{-/-}$ (0.89 ± 0.07 *vs* 0.81 ± 0.06). However, VS induced Akt activation was observed in $TNFR1^{-/-}$ mice (4.32 ± 0.1 *vs* 1.1 ± 0.06, $P<0.001$) (Fig. 5B).

3.3.3. Loss of ACh induced Protection on Cardiomyocytes

Since in vitro studies using ACh showed the peak effects on TNF-α protein at 12 h of hypoxia, we used this time as the optimal time for experiments with TNF receptors deficient cardiomyocytes.

MTT assay showed reduced viability of cells in response to ACh treatment in the $TNFR2^{-/-}$ cardiomyocytes ($48.1\pm2.1\%$ *vs* $34.2\pm2.6\%$, $P<0.05$) compared to cardiomyocytes from the wild type mice ($81\pm3.2\%$ *vs* $40.4\pm3.5\%$, $P<0.001$). In contrast, the protective effect of ACh was preserved in $TNFR1^{-/-}$ cardiomyocytes ($83.4\pm5.2\%$ *versus* $46.1\pm3.1\%$) which was comparable with the effect of ACh on cardiomyocytes from wild type mice (Fig. 6A).

ACh induced Akt phosphorylation was inhibited in hypoxia induced $TNFR2^{-/-}$ cardiomyocytes (0.77 ± 0.06 *vs* 0.74 ± 0.06). Even in the absence of hypoxia, ACh failed to upregulate the Akt phosphorylation in $TNFR2^{-/-}$ cardiomyocytes which is seen in the wild type cardiomyocytes (Fig. 6B).

As a result of Akt inhibition, ACh failed to inhibit caspase-3 activation (6.8 ± 1.1 versus 7.5 ± 1.1, fold increase compared to normal cells) in $TNFR2^{-/-}$ cardiomyocytes (Fig. 6C). Finally, as summarized in Fig. 7, activation of cell survival pathway by ACh was attenuated in $TNFR2^{-/-}$ cardiomyocytes as seen by less activation of pAkt and reduced mitochondrial membrane potential in response to hypoxia thus confirming that VS or ACh protects the cardiomyocytes through upregulation of cell protective TNF receptor subtype 2.

However, all the effects of ACh were preserved in the cardiomyocytes from $TNFR1^{-/-}$ mice under hypoxic and normoxic conditions (data not shown).

4. Discussion

The main objective of the present study is to determine the exact mechanism of action for the protection afforded by VS or ACh treatment against acute ischemia or hypoxic stress induced injury on the cardiomyocytes. Results from this study have demonstrated novel findings, that VS differentially regulated TNF-α and its cell protective receptor TNFR2 to protect myocardium against acute ischemic damage.

TNF-α is thought to contribute to the development of myocardial disease, with direct correlation between serum TNF-α and the severity and progression of heart failure [17–19]. Studies in transgenic mice with cardiac restricted over expression of TNF-α developed cardiac dilatation, abnormal calcium hemostasis, increased apoptosis, ventricular arrhythmias and early death [20,21]. In contrary to these findings, results from the recent clinical trials of anti-TNF-α therapy to treat heart failure were not successful [22,23], perhaps because of the possible dual role of TNF-α in attenuation and aggravation of cardiac injury [24]. Studies have demonstrated that TNF-α, could mimic ischemic preconditioning and has been shown to afford cardiac protection in various models of acute ischemia irrespective of the species specificity [25–27]. In another supporting report, Smith et al. [28] demonstrated the abrogation of the protective effects of ischemic preconditioning in TNF-α knockout mice. Therefore the role of TNF-α on heart remains to be highly controversial. In our study VS upregulated TNF-α mRNA, that could be observed as early after 30 min of LCA ligation and after 4 h of hypoxia. This upregulation of TNF-α is believed to act in the cell protective pathway, as VS treated hearts showed reduced myocardial infarct size and ACh treated cardiomyocytes exhibited improved cell viability under hypoxic conditions.

The source of TNF-α in the heart is considered to be mainly from the macrophages, but experimental studies from some laboratories have shown that adult mammalian heart can synthesize TNF-α mRNA and protein *de novo* after certain forms of stress [29,30]. An important study by Kapadia and colleagues [31] demonstrated the production of TNF-α in cultured cardiomyocytes both at the messenger level (after 30 min) and at protein level (after 75 min). They also suggested the possibility of translation of TNF-α mRNA in to biologically active protein. Our observations confirmed this theory, where, increase in TNF-α expression can be seen in the cardiomyocytes after 3 h of myocardial ischemia. Also in the in vitro conditions, ACh treatment increased the TNF-α protein from 2 h of hypoxia with the peak being observed at 12 h.

More interesting aspect in our study was the difference in the expression pattern of TNF-α by the cardiomyocytes during hypoxia.

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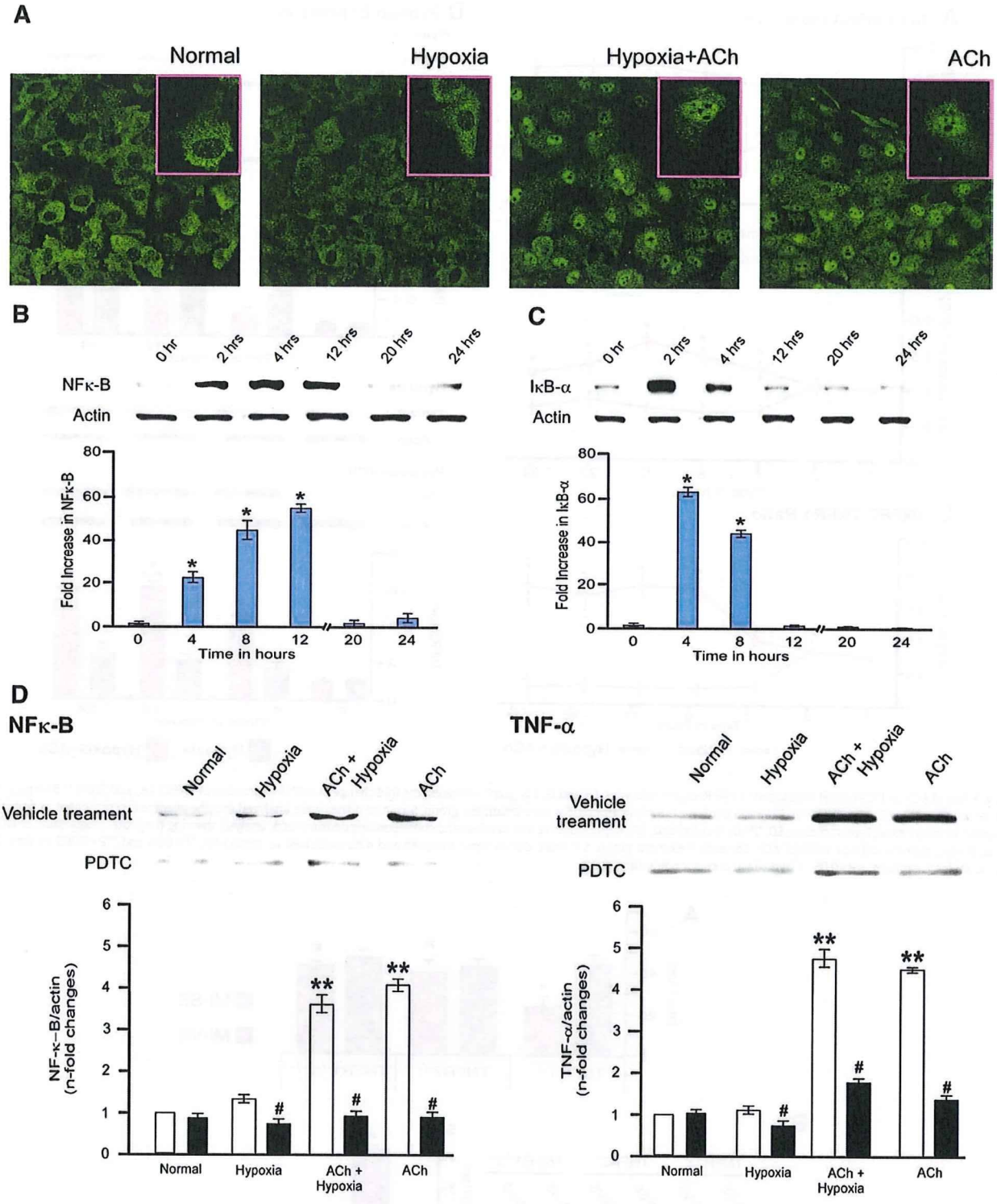

Fig. 3. Effect of ACh on Activation of Nuclear Factor Kappa-B and IκB-α. (**A**) Representative confocal images showing the expression of NFκ-B among the study group. ACh treatment induces nuclear localization of NFκ-B. (**B and C**) Representative immunoblot pictures and the corresponding densitometric analysis of the samples obtained at serial time intervals of hypoxia with ACh treatment. For each treatment group, 5 culture dishes were analyzed. Values are normalized by reference levels obtained from the samples of 0 h treatment and expressed as mean±SD. *$P<0.05$ vs 0 h. (D) Reperesentative immunoblot picture and corresponding densitometric analysis showing the expression of NF-κB and TNF-α after inhibition with NF-KB inhibitor PDTC (100 μM). Samples were collected at 12 h after hypoxia, as ACh treatment induced TNF-α expression was at peak at 12 h. Data expressed as fold changes compared to the vehicle-treated normal group and are mean±SD. n = 5 for each group. **P<0.005 vs normal; #P<0.01 vs corresponding vehicle-treatment.

ACh treatment increased the expression of TNF-α protein within few hours and after a peak at 12 h the level of TNF-α was downregulated. While in the untreated group, TNF-α expression was upregulated from 20 h after hypoxia (Fig. 2B). Therefore it is possible that acute upregulation of TNF-α by VS induces protective effects against acute ischemic insult.

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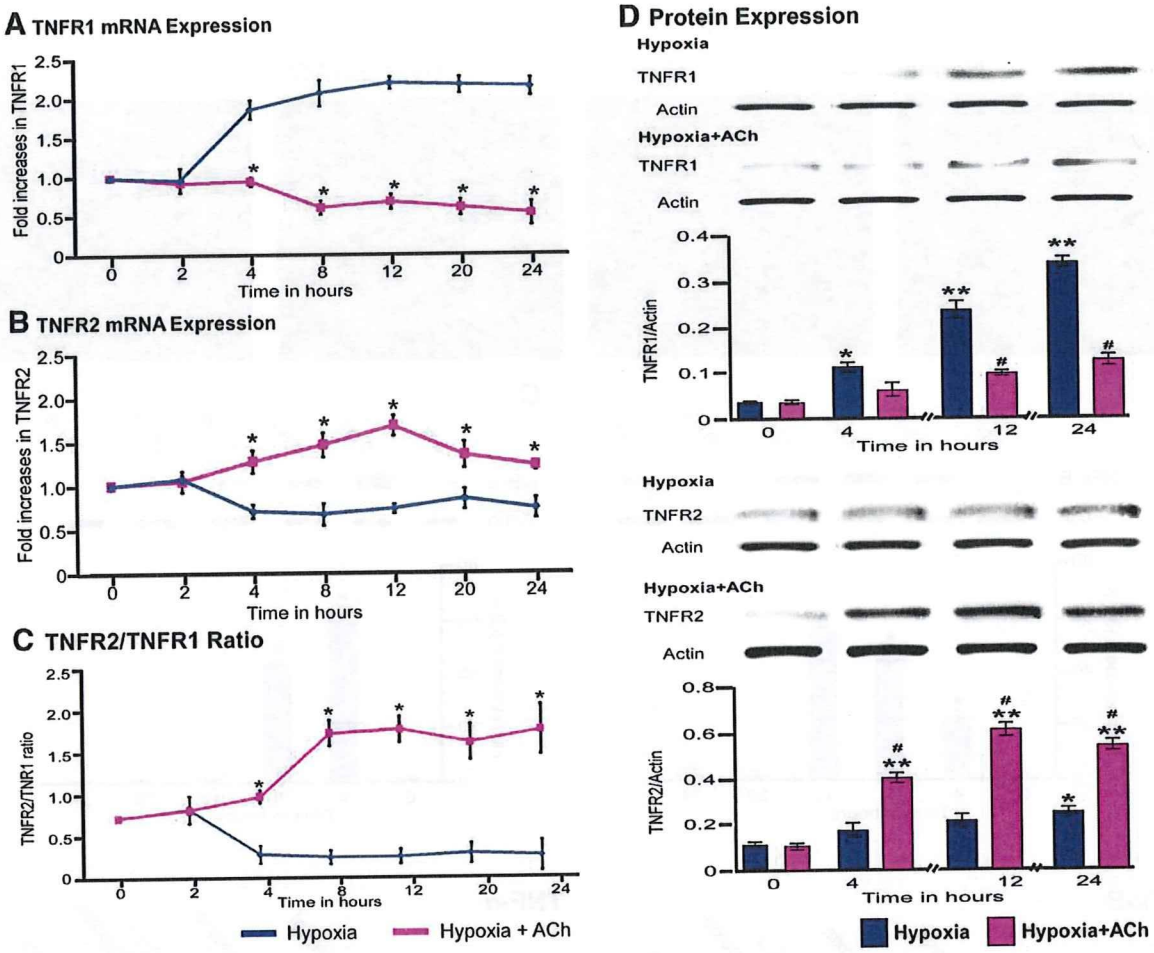

Fig. 4. Effect of ACh on Differential Regulation of TNF Receptor Subtypes. (**A and B**) Bar graphs showing the fold change in mRNA expression of TNFR1 (A) and TNFR2 (B) during hypoxia with or without ACh. (**C**) The TNFR2/TNFR1 ratio between the study groups. For each treatment group, 5 culture dishes were analyzed and the average is represented as densitometric analysis. Values are expressed as mean±SD. *$P<0.05$ vs hypoxia. (D) Representative immunoblot and corresponding densitometric analysis showing the protein expression of TNFR1 and TNFR2 during hypoxia with or without ACh. For each treatment group, 5 culture dishes were analyzed and data expressed as mean±SD. *$P<0.05$ and **$P<0.005$ vs time 0 of the corresponding treatment; #$P<0.05$ vs hypoxia at corresponding time point.

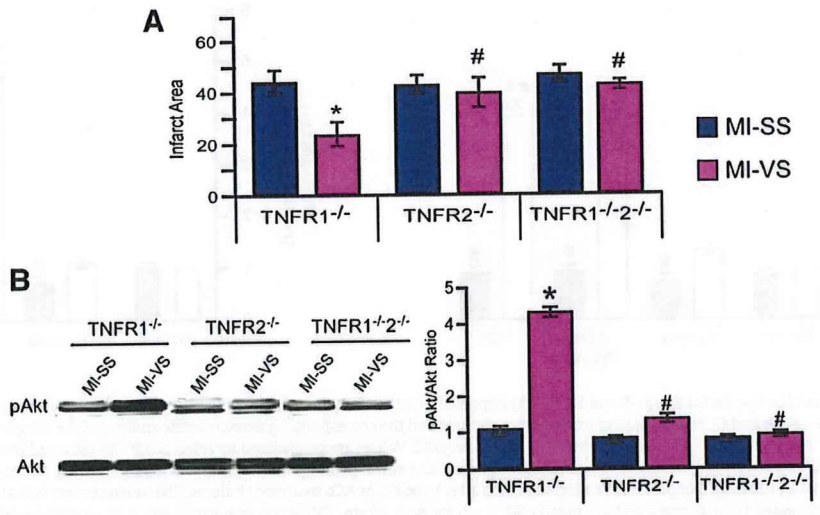

Fig. 5. Effect of VS on Myocardial Ischemia in TNF Receptor Deficient Mice. (**A**) Bar graph representing the area of myocardial infarction in TNF receptor deficient mice. n = 8 for each group. Data represented as the infarct area expressed as mean±SD. *$P<0.01$ vs MI-SS and #$P<0.01$ vs MI-VS of TNFR1$^{-/-}$ mice. (**B**) Reperesentative immunoblot picture (***left panel***) and densitometry results for pAKT expression in TNF receptor deficient mice after myocardial ischemia with or without VS. Values calculated as ratio between pAkt and Akt expression, and expressed as mean±SD. n = 5 in each group. *$P<0.01$ vs MI-SS and #$P<0.01$ vs MI-VS of TNFR1$^{-/-}$ mice. MI-SS – myocardial ischemia sham stimulation; MI-VS – myocardial ischemia vagal stimulation.

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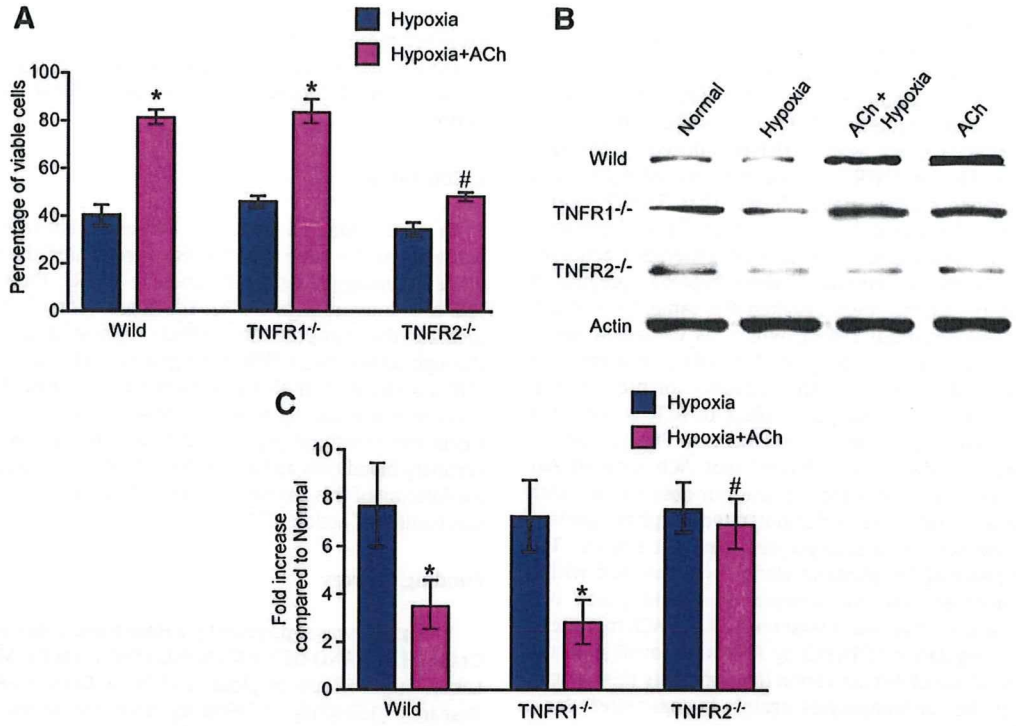

Fig. 6. Effect of ACh on TNF Receptor Deficient Mice Cardiomyocytes. (**A**) Bar graph showing the results of MTT assay in wild and TNF receptor deficient cardiomyocytes subjected to hypoxia with or without ACh treatment. For each treatment group, 5 culture dishes were analyzed. Values are expressed as mean±SD. *$P<0.01$ vs hypoxia, #$P<0.01$ vs hypoxia + ACh of wild and TNFR1$^{-/-}$ cardiomyocytes. (**B**) Representative immunoblot picture showing changes in the expression of pAkt under hypoxic conditions in the cardiomyocytes from wild and TNF receptor deficient mice with or without ACh treatment. ACh treatment failed to demonstrate protective effects in the TNFR2$^{-/-}$ cardiomyocytes. (**C**) Effects of ACh treatment on caspase-3 activity of wild and TNF receptors deficient cardiomyocytes. For each treatment group, 5 culture dishes were analyzed. Values are expressed as mean±SD. *$P<0.01$ vs hypoxia, #$P<0.01$ vs hypoxia + ACh of wild and TNFR1$^{-/-}$ cardiomyocytes.

To enlighten the underlying mechanisms behind ACh induced TNF-α production in cardiomyocytes we studied the activation of transcriptional factor NFκ-B. ACh treatment phosphorylated inhibitor kappa B-alpha (IκB-α), an inhibitor protein that keeps NFκ-B in the latent state in the cytoplasm. In this state NFκ-B is unable to induce TNF-α production [32]. Once IκB-α loses its complex state, NFκ-B is activated and translocated in to nucleus where it activates TNF gene transcription [33]. In our study phosphorylation of IκB-α was observed after 2 h of ACh treatment, followed by peak activation of NFκ-B at 8 h, while, the peak TNF-α activation was seen much later at 12 h of ACh treatment. Therefore it is conceivable, in the present study, NFκ-B could have been a source for the TNF-α synthesis by the cardiomyocytes after VS or ACh treatment (Supplemental Fig. 4). Although our study did not directly demonstrate the mechanism by which VS or ACh induces NFκ-B, previous studies have shown that neuronal NOS induce protection by activation of cGMP and NFκ-B [34,35]. Our earlier studies [4,36] also demonstrated the activation of NOS by ACh. Therefore, we believe that activation of NOS by ACh induced

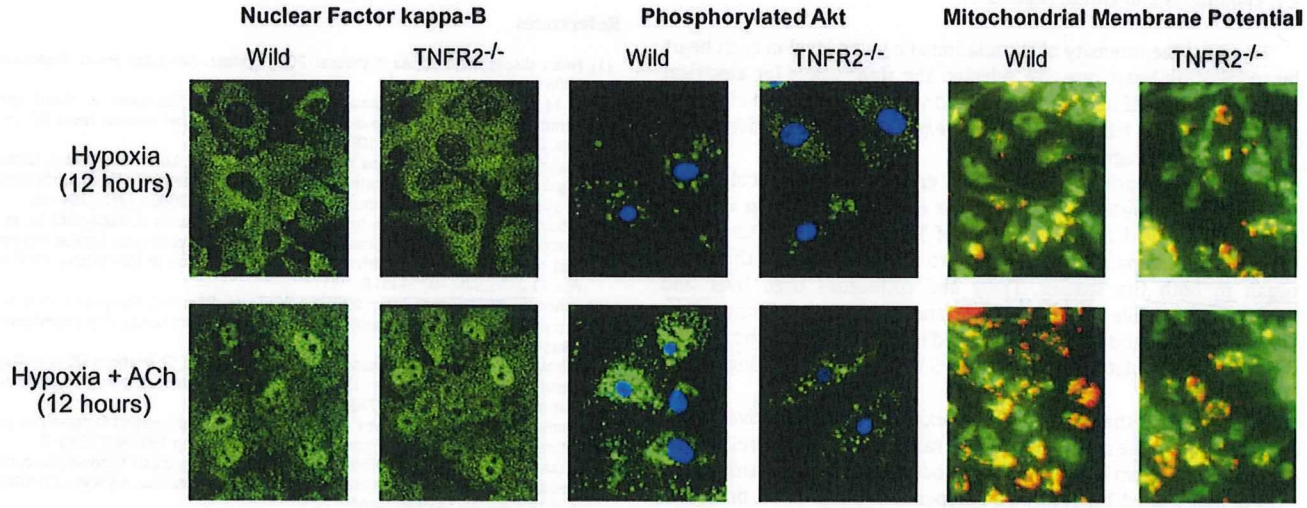

Fig. 7. Effect of ACh on cell survival signals in the TNFR2$^{-/-}$ Mice cardiomyocytes. Representative confocal image showing the defect in nuclear localization of NFκ-B, inhibition of pAkt activation and loss of mitochondrial membrane potential in TNFR2$^{-/-}$ dardiomyocytes after ACh treatment.

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cGMP which eventually lead to the activation of NFκ-B. As demonstrated by our results activated NFκ-B induced the activation of TNF-α.

The upregulated TNF-α acts through its two distinct receptors, TNFR1, which exerts devastating effects and TNFR2 which have a beneficial role on heart. Studies with TNFR1 knockout mice have demonstrated beneficial effect against TNF-α induced cardiomyopathy, while mouse lacking TNFR2 developed increased myocardial damage [11]. Further studies have shown that TNFR2 exerts neuroprotection in a phosphatidylinositol 3-kinase (PI3K) dependent manner that is counterbalanced by the neurodegenerative action of TNFR1 [10]. This was true in our study, where hypoxia upregulated TNFR1, while ACh treatment downregualted the same. In contrast, ACh upregualted the beneficial TNFR2, which, as explained above could have been a source for activating the Akt cell survival pathway by VS. Further, ACh also prevented the activation of proapoptotic caspase 3, suggesting that antiapoptotic effect of ACh is mediated through TNFR2/PI3K/Akt pathway (Supplementary Figs. 2 and 3). Even though earlier studies [4,37] showed that ACh induced Akt phosphorylation occurs at a more rapid phase, our observations with ACh treatment in serial time course demonstrated a biphasic pattern of Akt activation by ACh on cardiomyocytes (data not shown). The early or the first phase of Akt phosphorylation was observed within 1 h after ACh treatment and the delayed or second phase was observed after 6 h and maintained at least until 24 h of ACh treatment. We believe that upregulation of TNFR2 by ACh is responsible for the delayed or second phase of Akt activation that increases the capacity of ACh to protect the cardiomyocytes against hypoxic stress for a longer period of time.

With these results it becomes extremely necessary to confirm the essential role of TNFR2 in VS or ACh induced protection. To address this issue, we used the TNF receptor deficient mice as a direct evidence to demonstrate the TNFR2 mediated cardiac protection by VS. As expected, all the protective effects of VS and ACh were inhibited in the TNFR2 deficient and TNFR double knockout mice. However all the protective effects of VS and ACh were preserved in the TNFR1 deficient mice, confirming our hypothesis. Even though TNFR2 deficient cardiomyocytes showed a decreased activation of cell survival cascades, the expression of NFκ-B remained unaffected (Fig. 7), thus suggesting that activation of TNFR2 is independent of the NFκ-B activation. Further, the activation of NFκ-B could have increased the synthesis of TNF-α after ACh treatment and in the absence of TNFR2 receptor, this increased TNF-α binds with the TNFR1 receptor and acts in a cell destructive manner.

4.1. Limitations and Future Aspects

To adjust the intensity of stimulation at a given level in each heart by monitoring heart rate, we selected the right vagus for electrical stimulation. Therefore, it is not clarified whether or not the electrical stimulation of the left vagus nerve can exert the same effectiveness as that of the right vagus nerve.

Even though earlier study from the group of Tracy and colleagues [6] have demonstrated a decrease in the expression of TNF-α with VS, our study showed an upregulation of TNF-α after VS. The reason behind his discrepancy might be due to the differences in the target organ in both the studies. Tracy and colleagues used liver and macrophages while we used heart and cultured cardiomyocytes. However future studies could be of valid interest in demonstrating the difference in regulation of TNF-α by VS treatment depending on the target organs.

It is arguable that VS could have exhibited its protective effects mainly through the reduction of heart rate. However, our preliminary results with ex vivo heart perfusion model of vagal nerve stimulation [12] demonstrated bradycardiac independent effect of VS on TNF-α upregulation, after 3 h of myocardial ischemia (Supplemental Fig. 5). Therefore, we believe that VS exert protection independent of heart rate reduction in our experimental model, although this needs to be exploited more in detail.

Finally, use of cultured neonatal cardiomyocytes in this study could warrant the need for the use of adult cardiomyocytes in the future.

5. Conclusion

In conclusion, VS activates TNF-α in IκB-α/NFκ-B dependent pathway. At the same time VS also upregulated the beneficial TNFR2 while downregulating the destructive TNFR1, thereby directing the TNF-α to interact with TNFR2. This interaction of TNF-α with TNFR2 mediate the cytoprotective effects against acute ischemic injury through activation of PI3K/Akt signaling pathway. In addition to the TNF-a pathway, it is also worthwhile to be noted that VS could also exert its protection by activation of some neurotransmitters, such as vasoactive intestinal peptide (VIP), which is known to increase the coronary blood flow and act as the radical scavenger [reviewed in 38]. Exploration of this pathway could lead to the further insight in to the mechanism of action of VS.

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Disclosures

There was no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2010.03.007.

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Original article

Donepezil, an acetylcholinesterase inhibitor against Alzheimer's dementia, promotes angiogenesis in an ischemic hindlimb model

Yoshihiko Kakinuma [a,*], Mutsuo Furihata [b], Tsuyoshi Akiyama [c], Mikihiko Arikawa [a], Takemi Handa [a], Rajesh G. Katare [a], Takayuki Sato [a]

[a] Department of Cardiovascular Control, Kochi Medical School, Nankoku, Kochi 783-8505, Japan
[b] Department of Pathology, Kochi Medical School, Nankoku, Japan
[c] Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Suita, Japan

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ABSTRACT

Our recent studies have indicated that acetylcholine (ACh) protects cardiomyocytes from prolonged hypoxia through activation of the PI3K/Akt/HIF-1α/VEGF pathway and that cardiomyocyte-derived VEGF promotes angiogenesis in a paracrine fashion. These results suggest that a cholinergic system plays a role in modulating angiogenesis. Therefore, we assessed the hypothesis that the cholinergic modulator donepezil, an acetylcholinesterase inhibitor utilized in Alzheimer's disease, exhibits beneficial effects, especially on the acceleration of angiogenesis. We evaluated the effects of donepezil on angiogenic properties *in vitro* and *in vivo*, using an ischemic hindlimb model of α7 nicotinic receptor-deleted mice (α7 KO) and wild-type mice (WT). Donepezil activated angiogenic signals, i.e., HIF-1α and VEGF expression, and accelerated tube formation in human umbilical vein endothelial cells (HUVECs). ACh and nicotine upregulated signal transduction with acceleration of tube formation, suggesting that donepezil promotes a common angiogenesis pathway. Moreover, donepezil-treated WT exhibited rich capillaries with enhanced VEGF and PCNA endothelial expression, recovery from impaired tissue perfusion, prevention of ischemia-induced muscular atrophy with sustained surface skin temperature in the limb, and inhibition of apoptosis independent of the α7 receptor. Donepezil exerted comparably more effects in α7 KO in terms of angiogenesis, tissue perfusion, biochemical markers, and surface skin temperature. Donepezil concomitantly elevated VEGF expression in intracardiac endothelial cells of WT and α7 KO and further increased choline acetyltransferase (ChAT) protein expression, which is critical for ACh synthesis in endothelial cells. The present study concludes that donepezil can act as a therapeutic tool to accelerate angiogenesis in cardiovascular disease patients.

1. Introduction

Studies investigating the effects of vagal nerve stimulation (VS) on heart failure have suggested VS as a candidate for a therapeutic modality in heart failure because VS suppresses infarction-induced fatal arrhythmia and progression of ventricular remodeling [1,2]. However, the precise mechanisms remain to be fully elucidated.

To further investigate the underlying mechanisms of the VS effects, we have focused on disclosing the pleiotropic effects of acetylcholine (ACh) and revealed that ACh prevents cardiomyocytes from persistent hypoxia-induced cell death and have ultimately presented a new concept regarding ACh as a trophic factor. Our recent study demonstrated that ACh directly transduces cell survival signal through the muscarinic receptor, activates the PI3K/Akt/HIF-1α/VEGF pathway, inhibits collapse of mitochondrial membrane potential, and inactivates caspase-3 in cardiomyocytes subjected to hypoxia [3]. Because both survival and angiogenic pathways share common signaling molecules through HIF-1α/VEGF, these results prompted us to speculate the involvement of ACh in modulation of angiogenesis.

Furthermore, ACh transduces signals through nitric oxide (NO) production, and NO plays a key role in angiogenesis [4-8]. Specifically, according to our previous study, the NO donor *S*-nitroso-*N*-acetylpenicillamine activates the PI3K/Akt/HIF-1α pathway to increase VEGF expression in cardiomyocytes, and VEGF derived from cardiomyocytes accelerates tube formation in human umbilical endothelial cells (HUVECs), i.e., *in vitro* angiogenesis [4].

In contrast to these positive results, few *in vivo* studies have demonstrated the effects of systemically administered ACh because of its severe side effects including induction of bronchospasm and airway mucus hypersecretion. To circumvent this, we used donepezil, an acetylcholinesterase inhibitor and anti-Alzheimer's drug, that

* Corresponding author. Tel.: +81 88 880 2587; fax: +81 88 880 2310.
E-mail address: kakinuma@kochi-u.ac.jp (Y. Kakinuma).

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elevates local levels of ACh without such adverse effects [9,10]. In addition, we tested the effect of donepezil in a murine hindlimb ischemia model. To extensively investigate the effect of donepezil, we used α7 nicotinic receptor-deleted mice (α7 KO) suffering from impaired angiogenesis with characteristic mechanisms [11-13]. In the present study, we demonstrated a novel effect of donepezil on angiogenesis, i.e., acceleration of angiogenesis.

2. Materials and methods

2.1. Murine hindlimb ischemia model and donepezil administration

Male C57BL6/J mice (WT) ($n=45$) and α7 KO ($n=26$) aged 10–12 weeks were used. After anesthesia with pentobarbital sodium (30 mg/kg), the left femoral artery was completely ligated at its

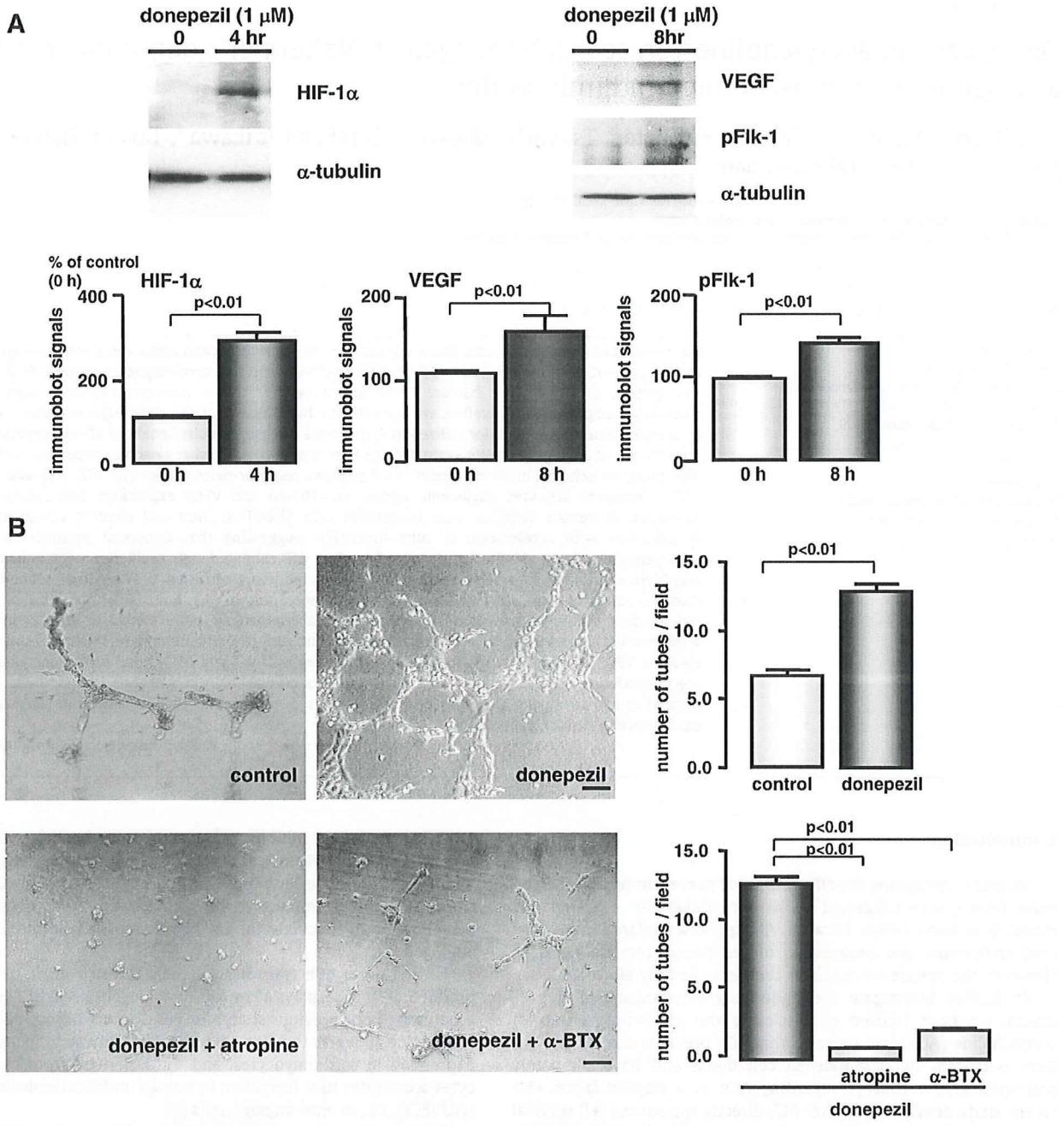

Fig. 1. Donepezil mediates angiogenic signals in HUVECs to activate tube formation. (A) Donepezil (1 μM) increased the HIF-1α protein expression level during normoxic conditions. This induction of HIF-1α was followed by increased VEGF expression and activated phosphorylation of the VEGF type 2 receptor Flk-1 in HUVECs ($n=3$). (B) Donepezil also accelerated tube formation in HUVECs (12.8±0.6 tube number per field vs. 6.7±0.4 in control, $P<0.01$, $n=3$). In contrast, donepezil-induced tube formation was inhibited by atropine (100 μM) or α-bungarotoxin (0.1 μM). Scale bar represents 50 μm. Representative data from 3 experiments are shown for each study. (C) ACh (100 μM) and nicotine (0.1 μM) increased HIF-1α and VEGF protein expression. (D) Tube formation in untreated HUVECs progressed within 24 h, and ACh markedly accelerated tube formation. ACh (100 μM) accelerated tube formation (16.8±0.9 in 100 μM of ACh vs. 7.3±0.5, $P<0.01$, $n=3$). ACh-induced acceleration of tube formation was partly suppressed by atropine (100 μM) (2.8±0.8, $P<0.01$, $n=3$) and α-bungarotoxin (0.1 μM) (2.7±0.6, $P<0.01$, $n=3$). Scale bars represent 100 μm. Representative data from 3 experiments are shown for each study (D).

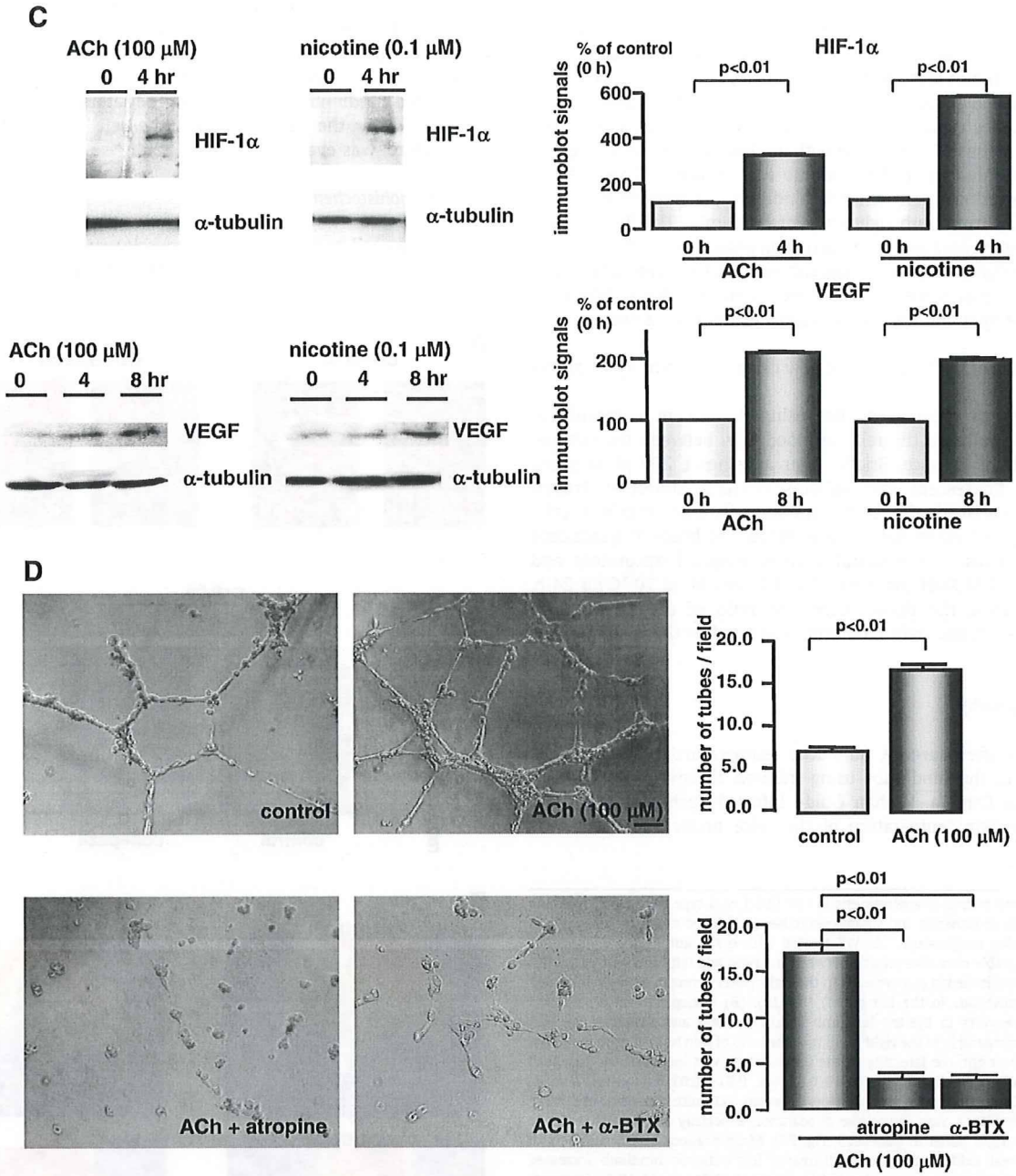

Fig. 1 (*continued*).

proximal end. Ligation was verified to be successful by pallor of the left foot. Donepezil (5 mg/kg/day) dissolved in drinking water (50 mg/L) was orally administered *ad lib* for 4 weeks. This dose was initially determined to clearly show the expected effects without producing adverse effects in the mice.

To investigate the involvement of cholinergic receptors on the effects of donepezil in terms of angiogenesis *in vivo* and to compare it with WT treated with donepezil alone, further donepezil-treated WT were divided into 3 subgroups ($n=6$–9 in each group) receiving one of the following treatments for 4 weeks: (a) α-bungarotoxin (14 μg/kg/day by i.m. on the flank), (b) mecamylamine (2.1 mg/kg/day i.m. on the flank), and (c) atropine (5 mg/kg/day p.o.) [14]. Another experimental study was conducted on α7 KO with a lower dose (0.083 mg/kg/day) using the same experimental schedule ($n=9$). This lower dose was comparable with that prescribed for patients. At the end of the treatment period, the heart and quadriceps femoris muscle were excised for experiments. Our preliminary study verified that even higher dose of donepezil, 5 mg/kg/day, does not down-regulate heart rate or blood pressure.

2.2. Angiography using indocyanine green dye

To functionally evaluate the effects of donepezil on murine angiogenesis, angiography was performed using indocyanine green (ICG) (Sigma-Aldrich, St. Louis, MO, USA), which clearly visualize tissue perfusion in the hindlimb. After anesthesia with pentobarbital sodium, both lower extremities were shaved. The field was illuminated by an LED-fluorescence imaging device, and ICG was admin-