ORIGINAL SCIENTIFIC ARTICLE

METFORMIN REVERSES THE EFFECTS OF ANGIOTENSIN 2 IN HUMAN MAMMARY ARTERIES BY MODULATING THE EXPRESSION OF NITRIC OXIDE SYNTHASES

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Received: 19.07.2022.
Accepted: 04.08.2022.

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UDK: 616.379-008.64-056.24:616.1
615.225.1.015
615.272.3.012.5
Ser J Exp Clin Res 2022; 23(3):201-207
DOI: 10.2478/sjecr-2022-0070

ABSTRACT

Angiotensin 2 impairs vascular function by activation of reactive oxygen species (ROS) production and development of endothelial dysfunction. Metformin, the first-line therapeutic agent for type 2 diabetes mellitus, has vascular protective properties, beyond its glucose lowering effects. The aim of the present study was to investigate the interaction between metformin and angiotensin 2 in human internal mammary arteries harvested from patients with coronary heart disease undergoing revascularization procedure, by evaluation of vascular function, reactive oxygen species (ROS) production and the gene expression of nitric oxide (NO) synthases (endothelial – eNOS, neuronal – nNOS and inducible – iNOS). To this aim, vascular samples were incubated with angiotensin 2 (Ang2, 12 h) with/without metformin (Metf, 10 μM) and used for ROS measurement (FOX assay), vascular reactivity in organ bath (contractility to phenylephrine, relaxation to acetylcholine, contractility to NG-nitro-L-arginine methyl ester/L-NAME) and RT-PCT studies. Acute incubation of the vascular rings with Ang2 impaired vascular reactivity (increase contractility, decrease relaxation), increased ROS production, supressed eNOS/nNOS and increased iNOS mRNA expression. Ex vivo incubation with metformin at a clinically relevant concentration reversed all these effects. These data suggest that Metformin might be useful in alleviating endothelial dysfunction by improving the endothelial-dependent relaxation and mitigating oxidative stress in clinical setting associated with cardiovascular disease regardless the presence of impaired glucose metabolism.

Keywords: *Human mammary arteries, angiotensin 2, metformin, endothelial dysfunction, NO synthases.*

INTRODUCTION

Increased generation of reactive oxygen species (ROS) contributes to the development of endothelial dysfunction mainly by limiting the bioavailability of nitric oxide (NO) and by inducing the activation of endothelial cells. Activation of both classic and local renin-angiotensin systems are recognized one of the most important contributors to the endothelial dysfunction in cardiovascular pathologies (1). In the vascular system the effects of angiotensin 2 (Ang 2) have been systematically investigated during the past decades and include vasoconstriction and prothrombotic effect, vascular remodeling with the proliferation of vascular smooth muscle cells and fibroblasts, increased expression of adhesion molecules in endothelial cells, and impaired nitric oxide (NO) generation/signaling (2-4).

Metformin, the first-line treatment for type 2 diabetes mellitus, used for more than 60 years, promotes also cardiovascular protection via pleiotropic, partially elucidated mechanisms (5). The beneficial effects in alleviating endothelial dysfunction have been reported in the setting of diabetes (6-8), metabolic syndrome (9), obesity (10, 11) albeit the mechanisms of action remains elusive. The mechanisms responsible for the regulation of endothelial cell functions by the drug in the setting of diabetes have been nicely covered by a recent review (12). However, direct anti-inflammatory effects have been reported irrespective of the diabetic status via a decrease in the secretion of proinflammatory cytokines and the markers of systemic inflammation (13). The anti-inflammatory properties of metformin have been unequivocally demonstrated in preclinical models, yet its use in the setting of diseases associated with chronic inflammation still remains elusive, as recently reviewed in ref. (14).

The present study was aimed to investigate the effects of metformin on vasomotor function, reactive oxygen species (ROS) level together with expression of nitric oxide (NO) synthases (endothelial, neuronal and inducible, eNOS, nNOS and iNOS) after Ang 2 stimulation of human internal mammary arterial samples harvested from patients with coronary heart disease subjected to coronary artery by-pass grafting.

PATIENTS AND METHODS

Study group

This study was conducted according to the World Medical Association Declaration of Helsinki and was approved by the Committee for Research Ethics of "Victor Babeș" University of Medicine and Pharmacy, Timișoara, RO (no. 04/28.02.2020 and 04p/17/12/2020). All participants provided a written informed consent.

The characteristics of patients included in the study are summarized in Table 1.

Table 1. Characteristics of the study group (n=7).

Parameter	Value (mean± SEM)
Age (years)	63.71±2.91
Sex (M/F)	6/1
Body Mass Index (kg/m^2)	27.75±1.48
Systolic Blood Pressure (mmHg)	136.28±3.36
Diastolic Blood Pressure (mmHg)	77.42±2.78
Heart Rate (b/min)	68.85±5.15
Erythrocyte Sedimentation Rate (mm/h)	31.57±9.79
Erythrocytes (mil/mm^3)	4.79±0.29
Hematocrit (%)	43.88±2.26
Hemoglobin (g/dL)	14.55±0.76
Leucocytes (*1000/mm^3)	8.42±0.83
Thrombocytes (*1000/mm^3)	248.71±11.67
Creatinine (mg/dL)	1.01±0.08
Uric Acid (mg/dL)	6.84±1.84
Total Cholesterol (mg/dL)	217.29±25.02
HDL-Cholesterol (mg/dL)	47.4±7.4
LDL-Cholesterol (mg/dL)	131.66±43.86
Fasting plasma glucose (mg/dL)	105.28±5.85
ALAT (U/L)	42.71±11.16
ASAT (U/L)	26±4.34
Na (mmol/L)	139.28±1.58
K (mmol/L)	4.24±0.13
CK (IU)	69.16±13.19
CK-MB (IU)	18.42±3.26
Main diagnostics	
Stabile angina pectoris, unstable angina pectoris, myocardial infarction, trivascular coronary artery disease, carotid atherosclerotic artery disease, mitral/tricuspid regurgitation, hypertension, heart failure NYHA II, hypercholesterolemia	
Preoperative medication	
Beta-blockers, loop diuretics, thiazide diuretics, ACEi, ARBs, statins, antiplatelet drugs, oral anticoagulants, nitrates, cerebral vasodilators	

Study design

Human internal mammary arteries were dissected under sterile conditions, cleaned, and incubated (12 h, 37°C in EBM culture medium with 0.1% BSA) in the presence or absence of Ang2 (100 nM) with/without Metf (10 μM). Subsequently, the vessels were used for vascular reactivity studies using the DMT myograph (contraction to Phenylephrine-Phe, relaxation to Acetylcholine-ACh and contractility in the presence of NG-nitro-L-arginine methyl ester - L-NAME, the classic eNOS inhibitor), ROS measurement (FOX assay) and gene expression of NO synthases (RT-PCR). We assessed the lowest concentration of Metf (10 μM) since the plasma therapeutic window of Metf in humans was reported to be between 10 - 40 μM (15).

Vascular reactivity studies

The human internal mammary arteries rings were suspended in the myograph chambers containing 5 ml of Krebs solution added with the cyclooxygenase inhibitor, diclofenac (10 μmol/L) aerated with 95% O2-5% CO2 gas mixture (pH 7.4, 37°C). Each vascular ring was fixed to a force transducer for the isometric force recording. Firstly, the vascular rings were stretched to a tension of 2 g and equilibrated for 1 h, and further exposed twice to 80 mM KCl. The concentration of Phe used for constriction was adjusted to obtain 80% of the KCl contraction. Subsequently, the endothelial-dependent relaxation to Ach and contractility to eNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 10μM) were recorded.

Assessment of oxidative stress

Hydrogen peroxide (H_2O_2) level was measured in human internal mammary arteries treated or not with Ang2 (100 nM) and Metf (10 μM) using the Ferrous iron xylenol orange OXidation (FOX) assay (PeroxiDetect Kit, Sigma Aldrich) as previously described (16-18). The production of H_2O_2 was calculated using a standard curve and the results were expressed in nmol H_2O_2/mg tissue/ h/.

Assessment of gene expression

Quantitative RT-PCR was performed in human internal mammary arteries rings. Total RNA was isolated (Aurum Total RNA Mini Kit, Biorad) and used for reverse transcription (iScript Advanced cDNA Synthesis Kit, Biorad). In order to determine gene expression, primers against were designed using sequence information from the NCBI database (5'→ 3'): eNOS-fw CTC ACC ATA GCT GTG CTG GCT TAC, eNOS-rv GAT GCA GGG CAA GTT AGG ATC AGG, nNOS-fw CTC CCG CCT CGG GCA AA CAG, nNOS-rv GTG CAC CCC GTT TCC AGC GT, iNOS- fw GCT CGC TTT GCC ACG GAC GA, iNOS-rv AAG GCA GCG GGC ACA TGC AA. The housekeeping gene (EEF2, eukaryotic elongation factor 2) and its primers were as follow (5'→ 3'): EEF2fw: GAC ATC ACC AAG GGT GTG CAG and EEF2rv: GCG GTC AGC ACA CTG GCA TA.

Statistics

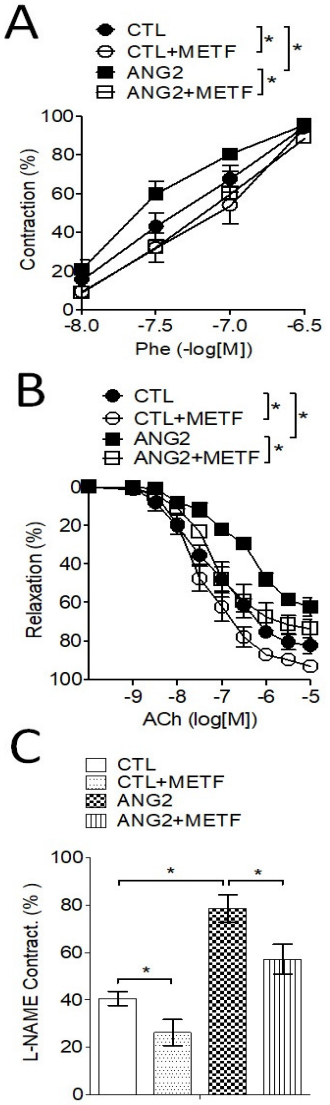
Statistical data processing was performed with the GraphPad Prism software Version 9.3.1 (GraphPad, USA). Data are presented as mean ± SEM and were analyzed using one-way ANOVA or Student t-test when appropriate. Data analysis of the dose–effect response curves was performed using the ANOVA F-test (comparisons of bottom and top values, EC50 and the Hill slope). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Metformin improved vascular function in human internal mammary arteries

In this study we investigated the interaction between metformin and Ang2 in human internal mammary arteries. We evaluated the vascular reactivity (contraction to Phe and the endothelium-dependent relaxation to ACh) in vascular rings stimulated or not with Ang2 in the presence *vs* absence of Metf. Ang2 impaired vascular function by increasing contractility and reducing relaxation and Metf was able to attenuate these effects (Fig. 1 A,B). The beneficial vasomotor effects of Metf were observed in all vascular rings (stimulated or not with Ang2), demonstrating the activation of the local renin-angiotensin system in the mammary arteries harvested from patients with coronary heart disease. As depicted in Fig. 1 A, B the magnitude of response appear to be higher in the Ang2-stimulated group, albeit the statistical significance was not evident due to the reduced number of samples. In the same experiments we measured the contractility response to eNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, Fig. 1C), as an indirect approach to assess the nitric oxide (NO) bioavailability.

Figure 1. Metformin improves vascular function in human internal mammary arteries stimulated of not with Ang2.



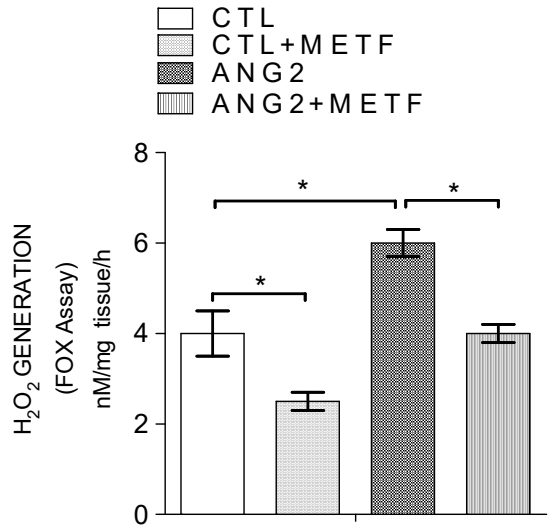
A. Phe-induced contraction; B. ACh endothelial-dependent relaxation; C. L-NAME induced contraction. n=7, *p<0.05

The contractility response was augmented by Ang2, but incubation with Metf was able to attenuate this effect suggesting the interference with the NO generation/signaling cascade as possible mechanism of vascular protection elicited by Metf.

Metformin reduced oxidative stress in human internal mammary arteries

To elucidate whether the improvement of vascular function was a consequence of ROS production modulation, in another set of experiments we investigated the effect of Metf on the H_2O_2 formation in human internal mammary arteries. As shown in Fig. 2, after *ex vivo* Ang2 stimulation, vascular H_2O_2 formation determined by the FOX assay increased. Co-incubation with Metf significantly attenuated this effect (Fig.2). Similar to the vasomotor effect, the drug was able to mitigate ROS prodution in CTL vessels (non-stimulated with Ang2) thus demonstrating that increased oxidative stress was present along with endothelial dysfunction at the level of mammary arteries used for grafting.

Figure 2. Metformin attenuated H_2O_2 generation in human internal mammary arteries stimulated of not with Ang2.



The H_2O_2 levels are expressed as nM/mg tissue/h, n=7, *p<0.05.

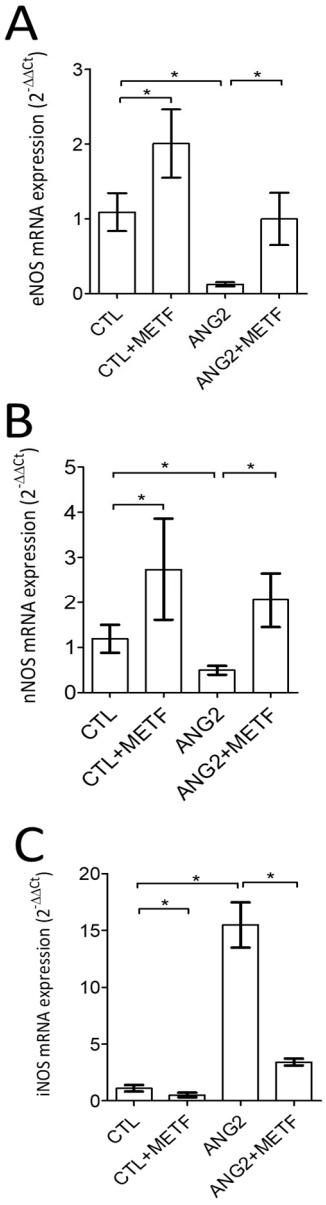
Metformin modulated the expression of nitric oxide synthases in human internal mammary arteries

As Metf was able to attenuate ROS generation and to improve vascular function, including reduction of NG-nitro-L-arginine methyl ester (L-NAME)-induced contractility, we further investigated whether Metf interfere with the mRNA gene expression of the enzymes responsible for NO generation in vessels. To address this aspect we performed qRT-PCR studies of the NOS isoforms (eNOS, nNOS, iNOS) in human internal mammary arteries stimulated or not with Ang2 and Metf.

In CTL samples, acute incubation with Metf elicited an increased expression of eNOS and nNOS and had no effect on iNOS.

Incubation of vascular segments with Ang2 in organic culture decreased expression of eNOS and nNOS and increased expression of iNOS, respectively. Metf was able to reverse all these effects (Fig. 3). Collectively, these experiments identify eNOS, nNOS and iNOS as targets of Metf in the human mammary arteries.

Figure 3. Metformin modulates NOS isoforms expression in human internal mammary arteries.
qRT-PCR mRNA gene expression.
A. eNOS, B. nNOS, C. iNOS, n=7, *p<0.05



DISCUSSIONS

This pilot study was purported to assess the effect of Metf on vascular reactivity, oxidative stress and NO synthases expression in human internal mammary arteries harvested during the revascularization procedure (aorto-coronary by-pass grafting) and stimulated or not *in vitro* with Ang2. The main finding of this study is that Metf was able to reverse the deleterious effects of Ang2 on vascular reactivity, ROS generation and gene expression of the enzymatic sources responsible for vascular NO generation.

Metformin has been introduced as diabetic medication in 1957 and considerable efforts have been made ever since to understand its beneficial effects at cellular and molecular level in order to argue for the drug repurposing in the cardiovascular pathologies (19). In the past decades the drug has also been systematically investigated for its protective effects in several other pathologies, such as: cancer, neurodegenerative diseases, inflammatory bowel disease, tuberculosis, polycystic ovarian syndrome, osteoporosis, periodontitis, and more recent, COVID-19 via modulating several signaling pathways such as: NF-κB, PI3K/AKT/mTOR, SIRT1/PGC-1α, NLRP3, ERK, P38 MAPK, Wnt/β-catenin, Nrf2, JNK as recently reviewed in ref. (20).

Currently, activation of AMP-protein kinase (AMPK) is considered the main molecular mechanism behind the protective effects of metformin in cardiovascular disease and cancer; moreover, the moderate increase in lactate, which act as a major energy shuttle in several lactate consumer-organs, has been recently proposed as being beneficial rather than deleterious after Metf administration (21).

The observation that Metf is able to improve the endothelium-dependent relaxation, an effect mediated by NO, has been reported 2 decades ago (22).

The mechanisms underlying the vascular beneficial effects of Metf have been studied in several experimental models. Thus, Davies et al firstly demonstrated that chronic administration of metformin in the wild-type C57BL6 mice enhanced eNOS activation and increased NO bioactivity (assessed as cyclic GMP), effects that were mediated via AMPK (since the effect was ablated in the AMPK-alpha1 knockout mice) (23). Three years later, Lefer's group demonstrated that chronic activation of AMPK with low-dose metformin elicited beneficial effects on cardiac function and survival in a murine model of heart failure; cardioprotection was mediated via increased eNOS phosphorylation in cardiac myocytes and was abolished in mice lacking functional eNOS (24).

Controversial data are available in the literature regarding the effects of Metf on NO release and NOS activity/expression in diabetic animals. Thus, while two studies reported the increase in eNOS expression (25, 26), no effect was observed by another group (27). As regarding the inducible NO synthase, the drug has been reported to negatively regulate the iNOS expression in vascular smooth muscle cells treated with TNFα (28). In a very recent data, Dawood et al demonstrated the activation of renal artery angiotensin 1 receptor, upregulation of endothelin-1 and iNOS, as well as downregulation of eNOS in a rat model of diabetic nephropathy, effects that were reversed by Metf (29).

In mesenteric arteries harvested from aged Otsuka Long-Evans Tokushima fatty (OLETF) rats, a type 2 diabetes model, a 4 week protocol of Metf administration both alleviated endothelial dysfunction and decreased superoxide production (30). We also demonstrated here that Metf is able to mitigate the oxidative stress, in particular the H_2O_2 generation. Important, in a previous study we reported that metformin does not have ROS scavenger properties (31), thus the reduction of ROS is the consequence of direct effect on ROS generation and/or on the antioxidant capacity, hypotheses that will be addressed in future studies.

As regarding clinical studies with Metf in humans, one of earliest studies in humans regarding the chronic beneficial vascular effects of administration of Metf (500 mg twice per day, 8 weeks) was performed in 33 non-diabetic women in which *in vivo* assessment of forearm (skin) microvascular function using laser Doppler imaging combined with iontophoresis was performed back to 2006. Endothelium-dependent- (but not independent) responses were improved by Metf *vs* placebo in women with angiographically normal coronary arteries vs placebo (32). In 2008, Meaney et al reported in the MEFISTO study that chronic administration of Metf (850 mg/day for 1 year) elicited a reduction in carotid intima–media thickness levels of carbonyls, dityrosines and AOPP and an increase in NO levels, thus indicating better endothelial function (33).

However, literature regarding the direct effect of Metf in human arterial samples is scarce. The novel observation of the present study is the direct modulation by acute incubation with Metf of all three NOS isoforms in human internal mammary arteries together with the above mentioned antioxidant effect.

CONCLUSIONS

In summary, Metformin, the first line therapy in diabetes, is able to alleviate endothelial dysfunction induced by Ang2 via the modulation of NOS expression and ROS production in human internal mammary arteries, the nonatherosclerotic repair arteries used for coronary bypass grafting.

Elucidating the molecular mechanisms underlying the Metf-NOS interaction in humans is required for a better management of the chronic vascular complications in cardiometabolic pathologies and/or of the acute vascular reperfusion injury.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted according to the World Medical Association Declaration of Helsinki and was approved by the Committee for Research Ethics of "Victor Babeș" University of Medicine and Pharmacy, Timișoara, RO (no. 04/28.02.2020 and 04p/17/12/2020). All participants provided a written informed consent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

Research supported by the university internal funds allocated to the Centre for Translational Research and Systems Medicine. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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