Original Article Metformin corrects RAGE overexpression caused signaling dysregulation and NF-κB targeted gene change

Xiang-Fang Chen^{1*}, Jun-Jie Zou^{1*}, Wei Tang^{1*}, Wei-Dong Lin², Liang-Liang Sun¹, Yi Bao¹, Hai-Yan Chen¹, Bei Zhang¹, Fei Ye¹, Zhi-Min Liu¹, Yong-Quan Shi¹

¹Department of Endocrinology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China; ²Department of Burns, Shanghai Fire Corps Hospital Chinese People's Armed Police, Shanghai 200443, China. *Equal contributors.

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Abstract: Background: Advanced glycation end products, known as AGEs, are substances that can be a factor in the development or worsening of many degenerative diseases, such as diabetes. The negative effect of AGEs has been noticed and studied on human keratinocytes but it is still poorly understood about the effect of diabetic drug on AGE caused skin damage despite the skin complications are critical problem of long course diabetes. Materials and methods: In order to study the effect of diabetic drug metformin on AGEs caused signaling dysregulation on human keratinocytes, we used lentiviral vector to overexpress the receptor for advanced glycation end products (RAGE) in immortalized human keratinocytes (HaCaT cells). Results: We showed that phosphorylation of p38, ERK1/2, JNK and Akt was dysregulated and all of them were corrected by metformin treatment. Moreover, the expressions of NF-kBp65 and downstream factors ICAM1, TNF- α , Cox-2, IL-6, and IL-1 β were also changed, in which the expression of those were rectified by metformin. Conclusions: This study is the first to show the therapeutic effects of diabetic drug metformin on AGEs caused signaling dysregulation on human keratinocytes.

Keywords: Human keratinocytes, RAGE, metformin, MAPK, PI3K/Akt

Introduction

Advanced glycation end products (AGEs) are substances generated from glycation reaction. They cause a lot of malfunction and signaling dysregulation and are seen as speeding up oxidative damage to cells [1, 2]. They are factors in the development or worsening of many degenerative diseases, such as diabetes, cardiovascular, atherosclerosis, chronic renal failure, and Alzheimer's disease [3, 4]. RAGE, the Receptor for Advanced Glycation End products is a 35 kD transmembrane receptor, and a member of the immunoglobulin superfamily, encoded in the Class III region of the major histocompatability complex [5, 6].

The involvement of RAGE in different diseases has been reviewed in great detail earlier [7, 8]. However, the detailed molecular mechanism of RAGE signaling, especially the involvement of various kinases and their substrates is not well understood. Most of the reported studies on RAGE signaling focus on activation of specific kinases like MAPKs, PI3K, PKC, GSK3 β and pro-inflammatory gene activation [9]. The pathogenesis is hypothesized to include ligand binding, upon which RAGE signals activation of nuclear factor kappa B (NF- κ B) and subsequently downstream genes involved in inflammation. It is important to identify the factors that facilitate the recruitment of specific kinases, along with their phosphoproteome, which can greatly enhance our understanding of the cellular responses to RAGE signaling.

It is the first-line drug of choice for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function. In recent years metformin was also shown to have multiple protective effects on cell models by distinctive mechanism [10, 11]. HaCaT cell is an immortal human keratinocyte line with their high capacity of dif-



Figure 1. Overexpression of RAGE in HaCaT cells. A: Real-time PCR detection of RAGE gene. Results represent mean \pm SEM of three independent experiments. B: ELISA assay of RAGE expression. Results represent mean \pm SD of three independent experiments. ***P* < 0.01 versus Naive group, ##*P* < 0.01 versus RAGE infection group.

ferentiation and proliferation in vitro [12]. Taken the advantages of short culture lifespan and low variations, we used lentiviral RAGE expression vector to make a cell model to primarily test the signaling change upon RAGE overexpression. Because of the interest of diabetes treatment, we also tested the effect of metformin together with RAGE overexpression. This study is the first to test the potential protective effect of metformin on human keratinocytes in a diabetic environment.

Materials and methods

Cell culture and treatment

Human HaCaT keratinocytes, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μ g/ml).

RT-PCR and real-time PCR

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions.

cDNA was synthesized by the reverse transcription kit (Qiagen, Suzhou, China) with 1 µg total RNA as template. Real-time RT-PCR was performed using SYBR Green kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) in an ABI LightCycler System (ABI-7300). Relative quantification of gene expression was determined by standard curve of each pair of primers and all relative concentrations were normalized to the expression of GAPDH as an internal RNA loading control. The primers used to amplify the coding region were as follows: 5'-CGGCTGGAATGGAAACTG-3' (forward) and 5'-TAGACACGGACTCGGTAG-3' (reverse) for RAGE.

ELISA

The cell surface expression of RAGE was tested with enzyme-linked immunosorbent assay (ELISA) kits (Pierce). Briefly, the samples described were incubated in 96-well plates coated with RAGE antibody for 30 min at 37°C. After 20 sec washes for 5 times, the samples were treated with enzyme working reagent for 30 min and with TMB One-Step substrate

Metformin protects human keratinocytes in diabetic environment



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Figure 2. MAPKs and PI3K signaling pathway alterations upon RAGE overexpression and metformin treatment. A: p-p38 and total p38. B: p-JNK and total JNK. C: p-ERK1/2 and total ERK1/2. D: p-Akt and total Akt. Results represent mean \pm SD of three independent experiments. ***P* < 0.01 versus Naive group, ##*P* < 0.01 versus RAGE infection group.



Figure 3. NF- κ B and downstream genes expression changes upon RAGE overexpression and metformin treatment. A: NF- κ Bp65 and total NF- κ B. B: ICAM1, TNF- α , Cox-2, IL-6 and IL-1 β expression changes. Results represent mean \pm SD of three independent experiments. **P < 0.01 versus Naive group, ##P < 0.01 versus RAGE infection group.

reagent for 15 min in the dark, and the reaction plates were read within 15 min in an ELISA plate reader (Labsystems, Multiskan, MS, Finland) at 450 nm.

Western blotting

Cells were washed twice with PBS and collected in lysis buffer with PMSF and protease and phosphatase inhibitors (Roche Diagnostics, Madison, WI, USA) to yield whole cell extracts. Cell lysates were resolved by SDS-PAGE and transferred to nitro-cellulose membranes according to standard procedures. The membranes were blocked with 5% non-fat milk and probed with primary antibodies for phosphorylated p38 (p-p38), p38, phosphorylated JNK (p-JNK), phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated Akt (p-Akt), and Akt at 4°C overnight. Antibodies against indicated protein were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). The membranes were incubated with the appropriate secondary antibody for 1 h room temp, and then the immunoreactivity was detected using enhanced chemiluminescence reagent. The relative intensities of the protein bands were analyzed by ImageJ software (National Institutes of Health).

Statistical analysis

Statistical analysis was carried out using either Student's t-test or one-way ANOVA for the comparison of more than two groups. Bonferroni's multiple comparisons test was used to conduct post hoc analysis. The data are presented as means \pm SD of at least three independent experiments. A value of *P* < 0.05 was considered statistically significant.

Results

Overexpression of RAGE in HaCaT cells

To investigate the possible effect of AGE in keratinocytes, several studies using AGE has been performed [13]. To achieve a high efficiency and steady expression of RAGE, in this study we used a lentiviral expression system to over-expression of RAGE human immortalized keratinocytes (HaCaT cells). The overexpression of RAGE was confirmed by both Real-time PCR (Figure 1A) and ELISA analysis (Figure 1B). Both mRNA (3.1-fold) and protein expressions of RAGE (>0.64-fold) are abundant compared with Naive group. Moreover, the expression of RAGE was 21.4 \pm 1.98% inhibited by metformin compared with RAGE overexpressed HaCaT cells without metformin treatment (Figure 1B).

MAPK and PI3K/Akt pathway changes are corrected by metformin

Because MAPKs and Akt are broadly connected to diabetic keratinocytes damage [14, 15], we first characterized the condition of p-p38, p-ERK1/2, p-JNK and p-Akt on RAGE overexpressed cells. We found the p38 was activated (Figure 2A) which was consistent with earlier study using AGE-BSA treating HaCaT cells. Meanwhile we found p-JNK, p-ERK1/2, and p-Akt were also activated (Figure 2B-D). Expectedly, we found that metformin administration corrected all the abnormality of activation. The corrected p-ERK1/2 was consistent with an earlier report of increased p-ERK1/2 in HaCaT cells by metformin treatment [16], but the effects on p-JNK and p-Akt were first showed on HaCaT cells by this experiment.

NF-κB and downstream cascade changes are corrected by metformin

We also checked the possible alteration of NF-κB signaling cascade on RAGE-overex-

pressed HaCaT cells together with the effects of metformin treatment. We found NF-kBp65 was increased upon the overexpression of RAGE, which indicating the involvement of NF- κ B in downstream of RAGE (**Figure 3A**) and was also corrected by metformin treatment. We next checked several NF- κ B downstream targets expressions that were related to inflammation and keratinocyte damage [17]. We found the expressions of ICAM1, TNF- α , Cox-2, IL-6 and IL-1 β are elevated. All of them were sensitive to metformin (**Figure 3B**). The effects of metformin on those factors on keratinocytes are all first characterized by our experiment.

Discussion

RAGE is expressed in tissues and cell types that are critical for immune surveillance including lung, liver, vascular endothelium, monocytes, and dendritic cells [18]. RAGE transduces signals to cause inflammation and serves as an adhesion receptor that inters with integrins and facilitates the recruitment of pro-inflammatory leukocytes to the sites of inflammation, further enhancing the inflammatory state [19, 20]. Moreover, Rage signaling also impacts the adaptive immunity, either directly or indirectly [21, 22]. These finds suggest that RAGE plays a role that is broadly involved in innate immunity and disease development. In this study, we used lentiviral vector to construct the overexpression vector for the receptor for advanced glycation end products (RAGE) in human keratinocytes (HaCaT cells), which revealed significantly increased in expression of RAGE both in mRNA (Figure 1A) and protein levels (Figure **1B**).

Metformin is an insulin-sensitizer drug widely used in conditions associated with insulinresistance such as type 2 diabetes. Metformin was shown to inhibit the expression of AGEs and RAGE axis contributes to diabetic nephropathy [23], and ameliorate tubular injury both in cell culture and diabetic animal model [24]. In the current study, we also found that the protein expression of RAGE was significantly inhibited in metformin treatment HaCaT cells, which was consistent with the previous results.

Among the known pathways that phosphoinsitide 3-kinases (PI3K)/Akt, and mitogen-activated protein kinase members (MAPKs) can be activated by RAGE [25, 26]. The latter group

includes Jun-N-terminal kinase (JNK), p38, and extracellular signal-regulated kinases (ERK) [27, 28]. AGE/RAGE axis has been reported to activation of PI3K/Akt in 3T3 adipocytes leading to their differentiation, obesity or predispose a person to diabetes [29]. In addition, AGE induces secretion of MMP 1, 3 and 13 in human osteoarthritic chondrocytes by interaction with RAGE and activation of JNK and p38 kinase [30]. On the contrary, AGE/RAGE signaling in the infiltrating macrophages causes phosphorylation of JNK, ERK1/2, and p38 and inhibition of JNK activity significantly enhances the effect of AGE on MMP9 secretion [31]. The data presented here demonstrated that PI3K/Akt and MAPKs pathway were significantly activated by RAGE overexpression in HaCaT cells (Figure 2A-D). In order to investigate the effect of metformin on the RAGE-mediated pathway, metformin was treated in RAGE overexpressed HaCaT cells. Thus, we hypothesized that metformin could either block or prevent the activated effects of RAGE on PI3K/Akt and MAPKs pathway. The present data showed that metformin was able to correct RAGE-induced activation of PI3K/Akt and MAPKs pathway in HaCaT cells.

In addition to the (PI3K)/Akt and MAPKs pathways, activation of RAGE also replays the cell surface signals to various intracellular pathways including the NF-B pathway, which is responsible for a myriad of transcriptional programs leading to the production of pro-inflammatory cytokines such as TNF- α and IL-1 β . Because the expression of RAGE itself is also controlled by NF-kB transcription factors [32], the activation of the RAGE-NF-KB signaling route results in an increased cell surface expression of RAGE, which amplifies the initial signal and further enhances inflammation [33]. While various cellular effectors have been successfully dissected and well studied, none of the cellular effectors in the RAGE signaling pathway has been identified and the molecular mechanisms of RAGE activates NF-KB remain unknown. Importantly, in the present study RAGE overexpressed HaCaT cells caused phosphorylation of NF-KB and metformin was able to completely abrogate the effect of RAGE similar to the results of previous studies (Figure 3A) [34]. Furthermore, inhibition of NF-KB was block differentiation of osteoclast cells through attenuation of TNF- α , IL-1 β and Cox-2 signaling. These findings agreed with our present report of RAGE overexpression increase of ICAM1, TNF- α , Cox-2, IL-6 and IL-1 β expression in HaCaT cells (**Figure 3B**). Nevertheless, metformin was remarkably inhibited the effects of RAGE on the expression of NF- κ B downstream factors.

In conclusion, our study is the first report testing the potential therapeutic effects of metformin in the background of diabetic environment on keratinocytes. RAGE overexpression affects the signaling of Akt and MAPKs and metformin corrects the levels of p38, p-ERK1/2, p-JNK and p-Akt. Moreover, our data demonstrate that NF-kB and its downstream targets are also activated by RAGE and metformin has more obvious therapeutic effect on the downstream targets in HaCaT cells.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Zhi-Min Liu and Yong-Quan Shi, Department of Endocrinology, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Huangpu District, Shanghai 200003, China. Tel: +86-21-55130726; Fax: +86-21-55130726; E-mail: zmliu_cz@hotmail. com (ZML); young.stone@163.com (YQS)

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