The Effects of Metformin, Aminoguanidine, and Pyridoxamine on Methylglyoxal Induced Cardiac Myocyte Injury

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Introduction

Cardiovascular disease is one of the many complications that can arise from diabetes[1]. Diabetic patients may undergo two main cellular changes regardless if glucose concentrations are properly maintained: advanced glycation of end (AGE) products and overwhelming oxidative stress within the cell. Although low levels of AGE are found normally at a basal level within the blood, diabetics have shown to have an increased level that may lead to atherosclerosis in vasculature[2].
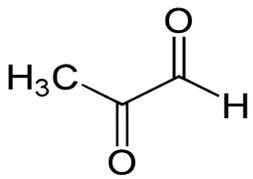
Methylglyoxal, a byproduct of glucose metabolism, has been shown to be elevated in the blood of diabetic patients and has been identified as an intermediate in the production of advanced glycation end products. Due to its structure, methylglyoxal is highly reactive with a wide range of cellular components. Specifically in the formation of AGEs, methylglyoxal modifies a select few amino acids and leads to final production of AGEs. The accumulation of these AGEs can cause inflammation, oxidative stress and cell death[3]. Similarly, methylglyoxal is able to produce high levels of reactive oxygen species (ROS) within the cell via mitochondrial modification which results in a loss of membrane potential and an increase in intracellular calcium concentrations. High levels of ROS are damaging to the cell and can lead to alteration in gene expression, inflammatory responses, structural changes, and cell death[4]. The effects and mechanism of methylglyoxal have not been fully elucidated.

Figure 1: Structure of methylglyoxal.

In order to reverse the effects of methylglyoxal, our study tests three compounds that have either been utilized in the treatment of diabetes or have been shown to decrease the amount of AGEs in the cell. Metformin, a common management medication for diabetics, works by lowering the level of endogenous methylglyoxal in the body, thus decreasing the quantity of AGEs produced and lessening the production of ROS[5]. Aminoguanidine hydrochloride and pyridoxamine dihydrochloride act to reduce levels of AGEs by inhibiting one of the intermediate steps, therefore decreasing the effects of AGEs and decreasing the amount of ROS as well[6,7]. Metformin, aminoguanidine hydrochloride, and pyridoxamine dihydrochloride utilize different mechanisms that interfere with the development of AGEs in the body, but these protective properties have not been tested with respect to cardiomyocytes.

In this study we compared the effects of metformin, aminoguanidine hydrochloride, or pyridoxamine dihydrochloride on methylglyoxal-induced cell damage and ROS production in H9C2 myoblast cells.
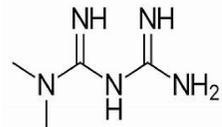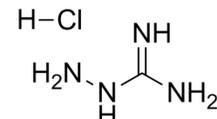
Figure 2: Structure of metformin.

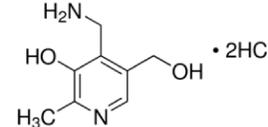Figure 3: Structure of aminoguanidine hydrochloride.

Figure 4: Structure of pyridoxamine dihydrochloride.

Hypothesis

We hypothesized that methylglyoxal reduced viability of H9C2 myoblast cells in a dose-dependent manner by increasing free radicals. By contrast, concurrently treated with both methylglyoxal and either metformin, aminoguanidine hydrochloride, or pyridoxamine dihydrochloride would increase cell viability accompanied with reduction of free radicals .

Methods

Measurement of cell viability: H9C2 rat myoblasts were seeded at 2×10^4 cells per well 24 hours prior to experiment. For testing the dose-response of methylglyoxal, cells were treated with varying concentrations (400 µM- 2000 µM) of methylglyoxal and incubated for 24 hours before analysis. To determine the protective effects, cells were treated with metformin (1-40 mM), aminoguanidine hydrochloride (250 µM- 2000 µM) and pyridoxamine dihydrochloride (0.1 µM - 15µM) in presence of methylglyoxal (1200 µM) and monitored for dose dependent effects after 24 hours. Cell viability was assessed using CCK-8 assay (Dojindo Molecular Technologies) after washing out all the compounds from medium. Moreover, cell morphology was observed using microscopy. Cell viability was expressed as the ratio of CCK readings to the non-treated control.

Measurement of intracellular ROS by DCFDA: After H9C2 rat myoblasts were seeded for 24 hours, cell media were replaced with 20 µM non-fluorescent dichlorofluorescein diacetate (DCFDA, Abcam) for 45 minutes to allow the dye to load into the cell. After washing out the unloaded DCFDA, the cells were incubated with media containing 1200 µM methylglyoxal with metformin (1-40mM) or aminoguanidine hydrochloride (250µm-8000µm). Inside the cell, DCFDA was deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into a highly fluorescent compound2', 7' – dichlorofluorescein (DCF). At 1 hour, 24 hours and >36 hours after treatment, fluorescence was measured at excitation (488 nm) and emission (527 nm) using a Fluroskan Ascent CF scanner (Thermo scientific). The change of ROS was expressed as the ratio to the baseline of non-treated control cells.

Statistical Analysis: All experiments were performed in triplicate and repeated at least three times. All values are presented as a mean ± SE. The significance was examined by ANOVA Fischer's PLSD. Values of $p < 0.05$ were considered statistically significant.
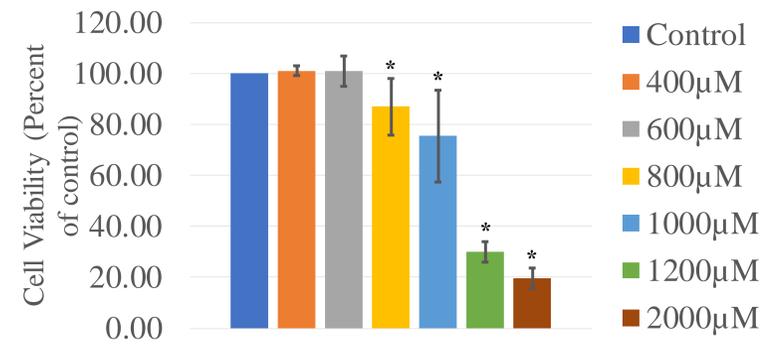
Results

Figure 5: Higher doses of methylglyoxal (800 µM-2000 µM) significantly reduced viability in H9C2 myoblasts when compared to the control (n=4, *: $p < 0.05$ vs. control).
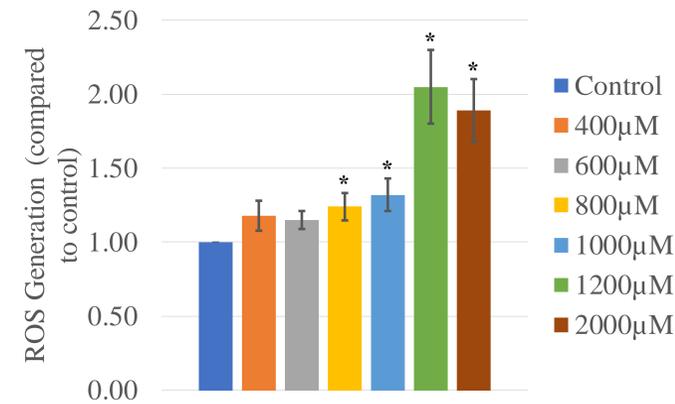
Figure 6: At 24 hours, higher doses of methylglyoxal (800 µM-2000 µM) showed significantly increased levels of ROS production compared to the control (n=3, *: $p < 0.05$ vs. control).
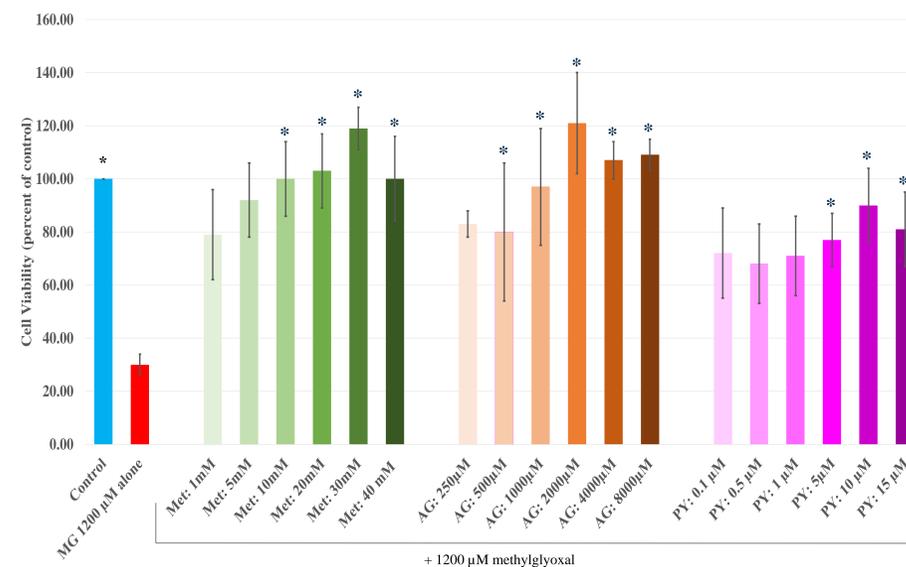
Figure 7: High doses of metformin , aminoguanidine hydrochloride and pyridoxamine dihydrochloride showed significant protection against methylglyoxal-induced cell death (n=7, *: $p < 0.05$ vs methylglyoxal).
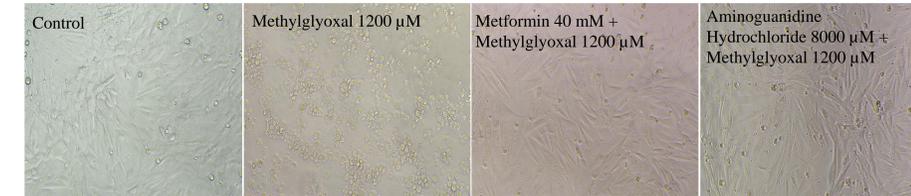
Figure 8: Representative microscopy of H9C2 cells (10X) under control, methylglyoxal 1200 µM, metformin 40 mM + methylglyoxal 1200 µM and aminoguanidine hydrochloride 8000 µM + methylglyoxal 1200 µM.
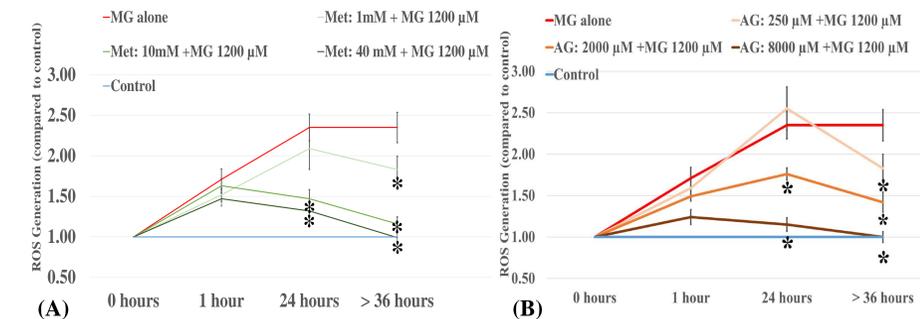
Figure 9: **(A)** Metformin showed a significant dose dependent reduction of ROS in H9C2 cells after 24 hours of incubation with methylglyoxal (n=3, *: $p < 0.05$ vs. control). **(B)** Aminoguanidine hydrochloride illustrated ROS reduction in higher doses after 24 hours when treated concurrently with methylglyoxal (n=3, *: $p < 0.05$ vs. control). Both metformin and aminoguanidine hydrochloride indicate significant protective effects against methylglyoxal- induced ROS.

Conclusions

We found that higher doses of methylglyoxal reduced cell viability in a dose- dependent manner which may be related to increased levels of ROS within the cell. However, when methylglyoxal was introduced with metformin, aminoguanidine hydrochloride, or pyridoxamine dihydrochloride, cell viability was significantly improved (p <0.05). Meanwhile, higher intracellular ROS caused by methylglyoxal was significantly decreased in the presence of metformin or aminoguanidine dihydrochloride. Future studies will investigate mitochondrial function and the possible mechanisms underlying the effects of methylglyoxal, metformin and aminoguanidine hydrochloride.

References

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Acknowledgements