Do Reduced Extracellular ATP Levels Contribute to the Development of Diabetes-associated Vascular Calcification?

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Arterial medial calcification (AMC) is a common condition of vessel hardening that leads to cardiovascular issues, many of which can be fatal. Vascular calcification was recently found to be a highly controlled process; however, the mechanisms of calcification are just beginning to be understood by researchers. While there is currently no effective treatment, researchers believe that phosphate along with other regulators play a large role in calcification. AMC is specifically seen in individuals with diabetes mellitus type II, which plagues a significant portion of our population. This investigation compared the effects of a high glucose environment on calcification, cell proliferation, and ATP release. Our high glucose environment was used to mimic the hyperglycemic state of diabetes patients. Glucose did not have an effect on calcification, cell number or cell viability in our model. However, glucose doubled ATP release per cell. Our results showed that models of calcification are difficult to compare since phosphate levels can vary so greatly. We saw no effect, which differed from other studies presumably because our phosphate concentration was much lower than others.

INTRODUCTION

Vascular calcification is the aggregation of calcium and inorganic phosphate (P_i), typically forming hydroxyapatite crystals, throughout the vascular system.[1] Arterial medial calcification (AMC) is characterized by the calcification of predominantly vascular smooth muscle cells (VSMCs) in the tunica media of blood vessels.[2] AMC is believed to increase the risk of cardiovascular death due to the reduction of aortic elasticity and cardiac efficiency.[3] Vascular calcification has conventionally been regarded as a passive degenerative process linked with aging.[4] However, recently researchers discovered similarities shared between AMC and bone formation.[5] In fact, VSMCs in a calcifying environment will undergo a phenotypic transdifferentiation stage and become similar to osteoblasts.[3,6] Moreover, recent studies have suggested that AMC is a highly controlled, cell-mediated process.[2,3,7,8]

Pyrophosphate is one of the key regulators of vascular calcification and bone mineralization. Inorganic pyrophosphate (PP_i) has been shown to inhibit calcification by blocking hydroxyapatite crystals from acting as nucleation sites for mineralization.[9] On the other hand, extracellular inorganic phosphate (P_i) has been seen to induce calcification of VSMCs.[8,10] The ratio of PP_i to P_i is also crucial to the regulation of AMC and bone mineralization.[11] In addition, it has been noted that the gain and loss of calcification inducers and inhibitors are critical to the regulation of AMC.[12]

Many proteins, enzymes, hormones, and genetic factors have been investigated in connection with vascular calcification.[13] Ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs) are highly researched enzymes that catalyze the hy-

drolysis of ATP to produce ADP and PP_i, effectively inhibiting calcification.[10] In fact, Prosdocimo (2009) showed that ATP is the main source of pyrophosphate in VSMCs, likely from the catalysis of NPPs. NPP1 is specifically significant in generating PP_i to reduce calcification in VSMCs.[14] In addition, tissue nonselective alkaline phosphatase (TNAP) catalyzes the breakdown of PP_i into P_i, which induces calcification. Additionally, the ankylosis protein (ANK) is a putative transporter whose role in calcification remains somewhat unclear.[6 9,14] The contrasting effects of these inducers and inhibitors, among others, play a large role in the homeostasis and regulation of vascular calcification.[14,15]

ATP is commonly known as the energy "currency" of the cell because it powers vital processes of the cell.[16] However, ATP is also an important extracellular signaling molecule that regulates vascular tone.[17] ATP binds to purinergic P2 receptors all around the body to adjust essential pathways such as cell proliferation, differentiation and function.[17] The P2 receptors are divided into 2 subcategories: P2X ion channels (P2X1-7) and P2Y G-protein coupled receptors ($P2Y_{1,2,3,4,6,11-14}$).[18] $P2Y_2$ has been specifically investigated for its role in VSMCs proliferation and calcification; however, its direct role in vascular calcification is not yet established. Although it regulates bone formation, $P2Y_2$ is suggested to not play a role in purinergic signalling of VSMCs.[19]

However, extracellular ATP has been seen to reduce vascular calcification both as an origin of PP_i and as a direct inhibitor of calcium phosphate deposition.[20] Both ATP and UTP have shown inhibitory roles in AMC, but researchers are skeptical that this effect is mediated by the $P2Y_2$ receptor in VSMCs.[19] Although some of the mechanisms involved are currently unclear, researchers are quite certain that ATP and PP_i are key suppressors of vascular calcification.[10]

Medial vascular calcification is commonly seen in patients with diabetes mellitus and chronic kidney disease.[21] Diabetes patients have very high frequencies of vascular calcification, which proceed at rapid rates.[3,22] Clinically, diabetes is characterized by an extensive hyperglycemic state.[23] While diabetes is linked with calcification, the mechanisms of this connection are poorly understood. Some research has suggested that high glucose concentrations increase calcification[24] while other experiments have shown insignificant effects of glucose on vascular calcification.[25] The relationship between calcification and diabetes is beginning to be researched, but it is far from being understood.

AMC is connected to considerable morbidity of cardiovascular disease and is a predictor of coronary heart disease.[21] Recent studies have shown that cardiovascular problems, such as vascular calcification, will become even more prevalent in the future. With an aging population becoming significantly more obese, the prevalence of diabetes is predicted to increase significantly.[26] So, vascular calcification is more important than ever to research and find an effective treatment. The aim of this study was to examine how glucose affects VSMCs in terms calcification, cell proliferation, and ATP release.

MATERIALS AND METHODS

Reagents

Alpha Minimum Essential Medium (αMEM), Dulbecco's Modified Eagle's Medium (DMEM), 0.5M glucose, 0.5M mannitol, 0.4M β-Glycerophosphate, 0.4M sodium diphosphate, trypsin (0.25% *w/v*), and Dulbecco's Phosphate Buffered Saline (PBS). All tissue culture reagents were purchased from Life Technologies (Paisley, UK), and all chemicals were purchased from Sigma Aldrich (Poole, UK), unless specified.

Cell culture

VSMCs were isolated from human umbilical cords (as described in Patel et al., 2016) and grown in DMEM supplemented with 10% fetal calf serum (FCS), Antibiotic-Antimycotic (ABAM), and L-glutamine. They were expanded in T175 tissue culture flasks in a 37°C incubator. Once confluent, they were passaged through trypsinization until passage 3 or 4. Once confluent again, VSMCs were trypsinized and separated into three groups: control, glucose, and mannitol. The control group had a baseline of 5.5mM of glucose (from the αMEM), which is within the normal plasma glucose concentration range (4-8mM) for humans[34]. The glucose group had an added concentration of 19.5mM glucose. The mannitol group was used as an osmotic control and had a concentration of 19.5mM mannitol. In these groups, VSMCs were plated onto 12-well plates with a density of approximately 5.0×10^4 cells/well or 24-well plates with a density of approximately 2.5×10^4. The cells were then cultured for up to 7 days in αMEM, supplemented with FCS, ABAM, and 1mM phosphate to induce calcification. VSMCs received half medium changes every 3 days.

Calcification assay

VSMCs were washed three times with PBS and incubated with 0.6M HCl at room temperature for 24 hours. The cal-

cium content was evaluated colorimetrically with a commercial kit (Sigma-Adrich, Poole, UK) by the chromogenic complex formed between calcium and *o*-cresolphthalein at 575nm. The protein content was measured colorimetrically with a Bradford assay at 450nm and 570nm. The calcium concentration was then normalized to the protein concentration.

ATP release assay

VSMCs were changed from αMEM to serum-free MEM to rid the VSMCs of colored media and FCS, both which could interfere with the luminometer reading. The cells were incubated for 1 hour in the new medium to allow for ATP release fluctuations due to the media change. Then a 25µl sample from each well was added to 25µl of ATP release reagent (CellTiter-Glo) in a large eppendorf. After 10 minutes, the ATP release was read on a luminometer, recorded, and normalized to cell number. This assay quantifies how much ATP is released, normalized to cell number, since ATP release is proportional to the luminescence. Note that since ATP release is highly variable and susceptible to changes caused by mechanical errors, outliers were eliminated from the data.

Cell viability and number assays

Lactate dehydrogenase (LDH) was measured in order to assess cell viability. LDH is present in very low levels in healthy cells, while dying cells release higher levels of LDH. A baseline of dead cells is established by this viability assay. The viability data was measured using the CytoTox 96® colorimetric cytotoxicity assay (Promega, UK, Southhampton, UK) at 495nm and then normalized to the cell number in the subsequent assay.

% viability =sample absorbance-minus blankcell lysis-minus blank x 100

For cell number, 1% Triton X-100 (lysis buffer) was added to the VSMCs to lyse the remainder of the live cells. The cells were scraped into the solution, and then a sample from each well was taken. LDH was measured for the total number of lysed cells, which gives approximate total number of cells from each well. Cell number was measured colorimetrically at 495nmn. Note that one hour before the assay, VSMCs were switched to serum free DMEM for the ATP release assay.

Statistical analysis

Results are expressed as means ± SEM for 6 or 12 replicates, which were performed at least three times using cells obtained from different umbilical cords. Data were analyzed using GraphPad Prism 7 software, and statistical comparisons were completed using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparisons test. A p value of ≤ 0.05 was considered statistically significant.

RESULTS

Glucose has no effect on calcification

To monitor the progression of calcification in the different groups, we analyzed calcium content of the VSMCs. The mannitol group was used as an osmotic control. Analysis of the calcium data revealed that there was no difference in the development of calcification between the control, glucose, and mannitol groups (Fig. 1). This suggests that glucose and mannitol do not affect calcification of VSMCs. In addition, the microscopy images (Fig. 2) illustrate the visual lack of differences between the three groups.

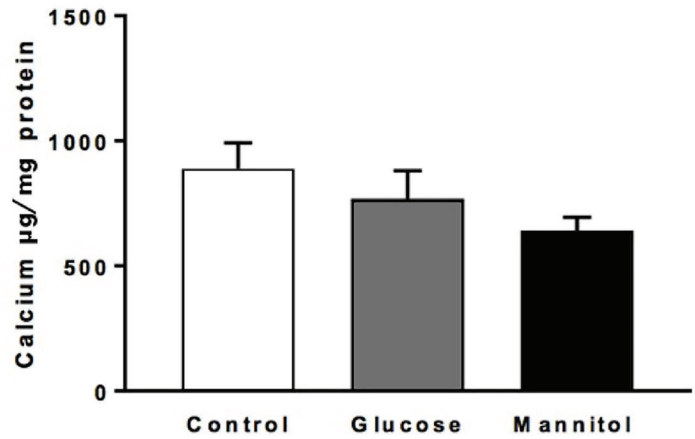

Figure 1. Calcium assay shows no effect. VSMCs showed no change between the three groups in calcium deposition normalized to protein content. Values are mean ± SEM (n = 6).

Glucose does not affect cell number but scarcely affects cell viability

We measured cell viability and cell number to ensure that the VSMCs were developing normally and to observe the effect

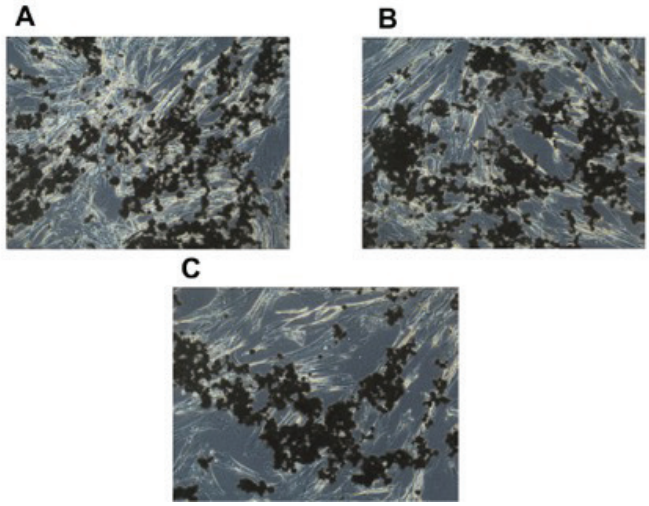

Figure 2. Microscopy images of vascular calcification show no visible differences. A portion of the well from each group was taken at x10 magnification. **(A)** Exhibits the calcification group, **(B)** shows the glucose group, and **(C)** displays the mannitol group. The three groups show no clear differences in proliferation of VSMCs or accumulation of calcium deposits.

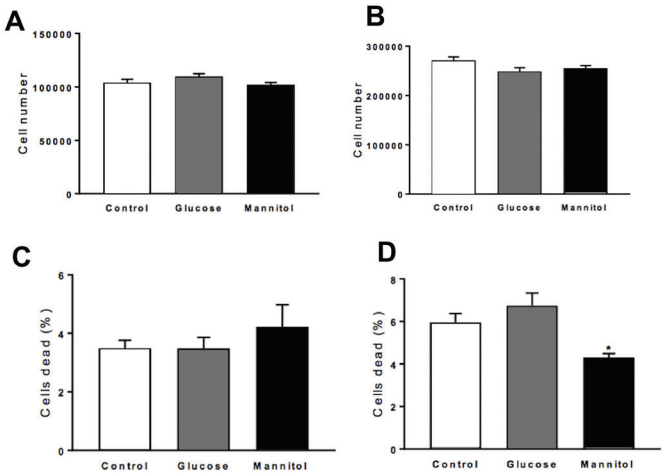

Figure 3. Cell number and cell viability shows limited effect. VSMCs showed no difference in cell number between the control, glucose, and mannitol groups on both day 4 **(A)** and day 7 **(B)**. In addition, VSMCs showed no difference in cell viability between the three groups at day 4 **(C)**. However, at day 7 **(D)**, the mannitol group had reduction of almost 40% in cell death compared to the control group, where p=0.0153. Values are mean ± SEM (n = 12), * = p<0.05.

of glucose on smooth muscle cell proliferation. Glucose had no effect on cell number at both day 4 (Fig. 3A) and day 7

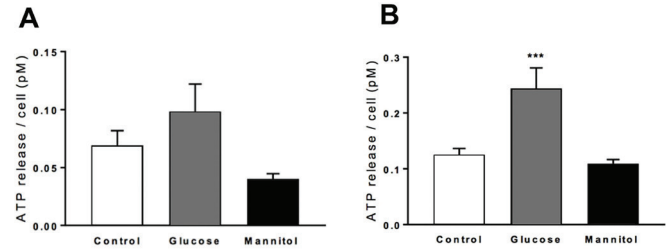

Figure 4. VSMCs show increased ATP release in glucose group. VSMCS exhibited no difference in ATP release normalized to cell number at day 4 **(A)**. However, at day 7 **(B)** the glucose group had doubled the ATP release per cell compared to the control group, where p=0.0006. Values are mean ± SEM (n = 12), *** = p<0.001.

(Fig. 3B). This suggests glucose does not affect cell proliferation. In addition, there was no effect of glucose on cell viability at day 4 (Fig. 3C). However, at day 7 mannitol had a 40% decrease in cell viability compared to the control group (Fig. 3D). As an osmotic control, mannitol did not affect the VSMCs at day 4, but by day 7 the interference caused decreased cell death. Glucose did not affect cell viability, suggesting it has mechanisms different from those of mannitol.

Glucose increases VSMC ATP release

We measured ATP release, as it is an inhibitor of vascular calcification and plays a key role in our understanding of the proliferation of VSMCs. There was no effect on ATP release per cell observed at day 4 (Fig. 4A). However, the ATP release per cell was doubled in the glucose group on day 7 (Fig. 4B). Increased ATP release in the glucose suggests that glucose is digested by the VSMCs. The progression of increased ATP release in the glucose group shows the gradual metabolism and release of ATP, which may regulate calcification. The mannitol group showed no effect on ATP release, suggesting the mannitol is not digested by VSMCs.

DISCUSSION

This study tested the effect of a high glucose environment on calcifying VSMCs. We found that glucose had no effect on levels of calcification and cell number and a limited effect on cell viability. However, glucose seemed to have a significant effect on ATP release normalised to cell number.

Our results showed there was no difference in calcium content between the three groups. Unlike previous studies,[24,27] we found glucose had no effect on calcification. There

are a mentioned used 10mM β-Glycerophosphate (βGP) to induce calcification, while our study used only 1mM β-GP. This 10-fold difference in phosphate could play a role in the discrepancies between our findings and previous ones since phosphate is a key inducer of calcification. Our study used only 1mM phosphate because it is more physiologically relevant at that level.[6] In fact, serum phosphate levels over 3mM are extremely rare in the human body, even in severe hypophosphatemia.[28]

Secondly, phosphate stresses the cells, which could contribute to the increased calcification.[29] The prior experiments also may have caused increased calcification because the VSMCs were more stressed in this high glucose and high phosphate environment, causing further calcification. Finally, we were limited to three replications of the experiment, while the previous studies had possibilities for more. So, it is possible that we could garner similar results if the experiment was repeated several more times. However, since all three of the replicates were very similar, it is more likely that the difference in β-GP plays a key role in the contrasting data. In addition, other researchers have criticized the variability in phosphate concentration as a limitation of many vascular calcification studies.[28,30]

We did not observe the same effects on cell number and cell viability as we expected based on previous research. Chen et al. (2006) described the effects of glucose on VSMCs increasing growth and proliferation. However, we found no effect of glucose on cell number or cell viability on both days 4 and 7. One reason for the discrepancy in our results might be due to the 10-fold difference in phosphate between our experiment and previous ones. As mentioned above, the high phosphate and high glucose environment in those studies may have caused increased cellular stress. Cellular stress can result in multiple different cellular responses including cell growth and proliferation.[31] So, it is possible that in a high phosphate and high glucose environment, VSMCs are stressed and induce a cell proliferation pathway. However, in solely a high glucose environment, it is possible that the VSMCs were not stressed enough to activate these proliferation pathways, resulting in no effect. However, we did find a significant decrease in cell viability in the mannitol group on day 7. It is possible that the high osmolarity and lack of metabolism in the mannitol group caused an increase in cell death.

Prosdocimo et al. (2010) suggested that ATP release plays a specific role in a calcifying environment. ATP release is believed to be coupled with NPP1 activity, effectively releasing ATP simply as the substrate for NPP1 to produce extracellular PP_i. In addition, ATP is thought to be broken down so efficiently by NPP1 that there is only a minimal increase in ATP release. However, our results did not show the same effects.

Our results showed an uptrend in ATP release in the glucose group at day 4 and a significant increase in ATP release at day 7. While this is not the effect that we expected, there are a couple explanations as to what may be happening. Firstly, the prior study used 3 or 5mM phosphate, which could have altered some pathways in the VSMCs since it is a much more hyperphosphatemic environment. Secondly, it is possible that the uptrend in cell death in the glucose group at day 7 contributed to the increase in ATP release. When cells die, they release all their contents through a process of necrosis, so it is possible that necrosis played a role in the increase in ATP release.[32] Thirdly, it is possible that the increase in ATP release is simply due to the metabolism of glucose, which is increased in that group. However, it is also feasible that the change is due to another mechanism unaware to us.

When considering these results together, we wonder why the increase in ATP release in the glucose group seemed to have no effect on calcification. Since ATP is known to be an inhibitor of vascular calcification, we would expect a decrease in the calcification of the glucose group; however, we found no effect. This begs the question whether the increase in ATP release was enough to be physiologically significant since it did not seem to affect calcification. In addition, we must consider the other roles that ATP may be taking in purinergic signaling of VSMCs. Since researchers have not yet established the role of most P2 receptors in vascular calcification,[33] ATP could have a variety of signaling paths in VSMCs.

In addition, increased apoptosis is connected with increased vascular calcification as apoptotic cells can serve as spots for further nucleation.[12,13] In our results, this was represented in the mannitol group. At day 7, mannitol had decreased cell death as well as a downtrend in the calcium content. Although this is not the most concrete evidence of this effect, we do see the same trend as prior studies.

Although our study was a limited experiment with only three replications, we were able to draw a couple conclusions from our data. Glucose did not appear to have the same effect on calcification, cell number, cell viability, and ATP release at 1mM phosphate as it does at higher levels. Although it is a limitation that experiments with different levels of phosphate cannot reasonably be compared, this experiment set a baseline for 1mM phosphate since most studies

have not used such low levels. In addition, we found that glucose increases ATP release but through a mechanism that we were not able to discern from this study. Furthermore, the increase in ATP release did not seem to have an effect on calcification, which is most likely due to another mechanism that we are not aware of from this study. However, this experiment opens up many doors for further studies. qPCR could be run on the three test groups to see which genes are being expressed. Moreover, we could measure at more time points like days 2, 4, 7, and 10 in order to get a better picture as to what is happening. In addition, we could add high fructose as a fourth test group since it is extremely common in food, especially foods that are known to contribute to diabetes type 2.

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