BMB
Reports

BMB Rep. 2020; 53(11): 588-593
www.bmbreports.org

Activation of pannexin-1 mediates triglyceride-induced macrophage cell death

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The accumulation of triglycerides (TGs) in macrophages induces cell death, a risk factor in the pathogenesis of atherosclerosis. We had previously reported that TG-induced macrophage death is triggered by caspase-1 and -2, therefore we investigated the mechanism underlying this phenomenon. We found that potassium efflux is increased in TG-treated THP-1 macrophages and that the inhibition of potassium efflux blocks TG-induced cell death as well as caspase-1 and -2 activation. Furthermore, reducing ATP concentration (known to induce potassium efflux), restored cell viability and caspase-1 and -2 activity. The activation of pannexin-1 (a channel that releases ATP), was increased after TG treatment in THP-1 macrophages. Inhibition of pannexin-1 activity using its inhibitor, probenecid, recovered cell viability and blocked the activation of caspase-1 and -2 in TG-treated macrophages. These results suggest that TG-induced THP-1 macrophage cell death is induced via pannexin-1 activation, which increases extracellular ATP, leading to an increase in potassium efflux. [BMB Reports 2020; 53(11): 588-593]

INTRODUCTION

Atherosclerosis is a disease characterized by the narrowing and hardening of arteries due to the formation of lipid-laden plaques, often leading to ischemic cardiomyopathy and stroke (1). Since the abnormal accumulation of lipoproteins, especially oxidative low-density lipoproteins (Oxi-LDL), is considered the first step in the pathogenesis of atherosclerosis, many studies have been directed towards understanding the characteristics of Oxi-LDL and oxidative stress (2, 3). Multiple lines of evidence support that elevated TG levels are associated with an increased risk of atherosclerosis and coronary heart disease (4, 5). We have previously demonstrated that TGs induced the macrophage death via caspase-1 and 2 (6, 7). These studies suggested a putative mechanism by which TG contributes to the development of atherosclerosis. However, the mechanism by which TG triggers the caspase cascade in macrophages remains elusive.

Potassium efflux occurs during cell death due to loss of ionic homeostasis. Furthermore, dysregulation of potassium efflux is one of the causative factors of caspase-dependent cell death (8). For example, the potassium ionophore valinomycin can induce potassium efflux, which triggers caspase-3 activation and subsequent cell death in CHO cells (9). Furthermore, α-toxin-mediated caspase-2 activation and cell death were suppressed on preventing potassium efflux in HeLa cells, indicating that potassium efflux is a prerequisite for caspase-2-mediated cell death (10). Many studies have speculated on the mechanisms by which potassium efflux is regulated. One of the well-known mechanisms for increasing potassium efflux is the opening of the ATP-sensitive potassium channel (11). It has also been reported that ATP-sensitive potassium channels open during the initial phase of myocardial ischemia, one of the consequences of atherosclerosis (12).

Pannexins are large pore-forming channels and consist of three family members (pannexin-1, 2, and 3). Since pannexin-1 is ubiquitously expressed ATP-permeable channel and is involved in a variety of cellular processes, including cell death, it has attracted significant attention in research (13). In the current study, we investigated the involvement of potassium efflux in TG-induced macrophage cell death. We report that TG induces an increase of extracellular ATP via pannexin-1, which in turn activates ATP-sensitive potassium channels, resulting in an increase in potassium efflux and subsequent activation of the caspase cascade in THP-1 macrophages. These results suggest that TG-induced cell death of macrophages is mediated by the activation of pannexin-1.

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https://doi.org/10.5483/BMBRep.2020.53.11.179

Received 27 August 2020, Revised 10 September 2020, Accepted 28 September 2020

Keywords: Cell death, Pannexin-1, Potassium efflux, THP-1 macrophages, Triglyceride (TG)

ISSN: 1976-670X (electronic edition)

RESULTS

Potassium efflux is involved in TG-induced THP-1 macrophage cell death

Though previous studies have reported that TG-induced cell death is mediated by caspase-1 and 2 (6, 7), the mechanism by which they are activated is not fully understood. Several studies have reported that the release of intracellular potassium ions activates caspases (14, 15). Therefore, this study investigated whether potassium efflux is involved in TG-induced caspase-mediated THP-1 macrophage death. When extracellular potassium concentration was measured in PMA-differentiated THP-1 macrophages (incubated with or without 1 mg/ml of TG for 24 h), it was found to be higher in TG-treated macrophages (Fig. 1A). Furthermore, when macrophages were treated with glyburide, an inhibitor of ATP-sensitive potassium channels, TG-induced cell death was restored in a dose-dependent manner (Fig. 1B). When the extracellular concentration of potassium was increased via treatment with 25 mM KCl to prevent the release of potassium ions, cell viability was recovered (Fig. 1C). Suppression of potassium efflux by treating macrophages with glyburide reduced the levels of cleaved PARP and several caspases (caspase-3, 7, 8, and 9) that are known to be responsible for TG-induced macrophage death (Fig. 1D). When THP-1 macrophages were incubated with TG in the presence of glyburide, treatment with glyburide decreased the activity of caspase-1 which is reported to induce the activity of caspase-3, -7, -8, and -9 (7) (Fig. 1E and 1F). Treatment with glyburide also reduced the activity of caspase-2, a known upstream molecule of caspase-1 (7), which increased upon TG treatment (Fig. 1G). These results demonstrate that potassium efflux induces TG-stimulated macrophage death via the activation of caspases.

The increase in extracellular ATP leads to TG-triggered macrophage cell death

It has recently been reported that an increase in extracellular ATP affects ATP-sensitive potassium channels, thereby increasing potassium efflux (16). Therefore, to determine if extracellular

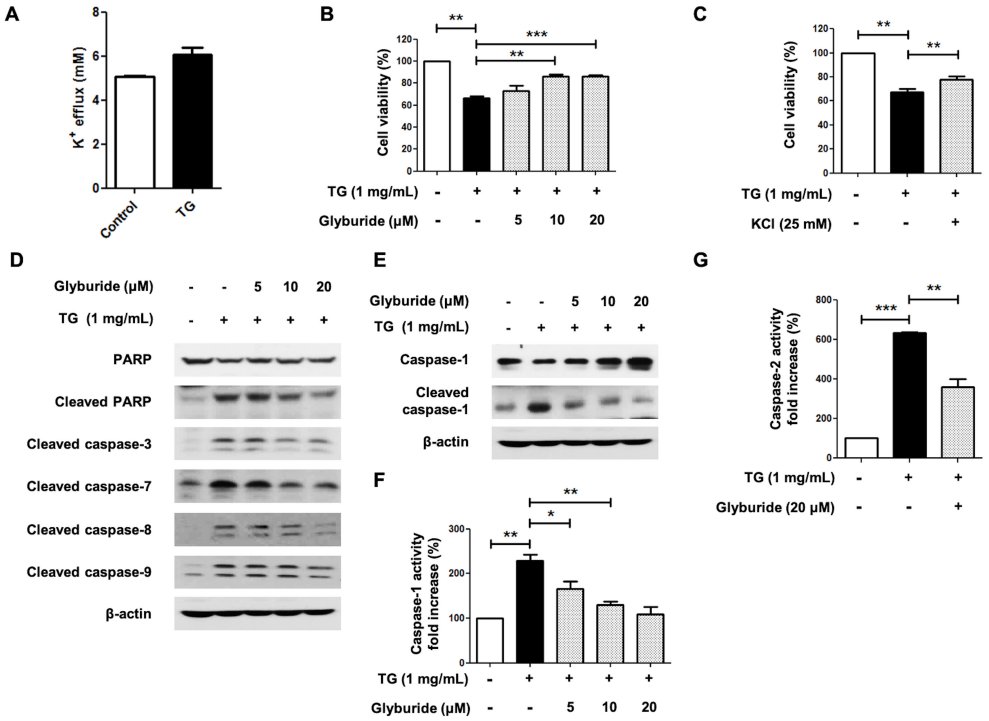

Fig. 1. Potassium efflux is involved in TG-induced THP-1 macrophage cell death. (A) THP-1 cells were differentiated with PMA and incubated with or without TG for an additional 24 h. Extracellular potassium concentration was measured by atomic absorption spectrometry. (B) THP-1 macrophages were incubated with TG in the absence or presence of the potassium efflux inhibitor, glyburide. Viable cells were enumerated by the trypan blue dye exclusion assay. The number of viable cells in THP-1 macrophages without TG treatment was set as 100%. (C) THP-1 macrophages were incubated with TG in the presence of KCl for 24 h and the trypan blue exclusion assay was performed. (D) The cleaved form of PARP, and the cleavage of caspase-3, -7, -8 and -9, were detected by Western blotting. (E) The cleavage of caspase-1 was detected by Western blotting. (F) THP-1 macrophages were incubated with TG in the presence of glyburide for 24 h, after which caspase-1 activity was assessed. The absorbance of THP-1 macrophages without TG treatment was set as 100%. (G) THP-1 macrophages were incubated with TG in the presence of glyburide for 24 h and caspase-2 activity was assessed. All data are expressed as the mean ± SEM of three independent experiments. P-values were determined with Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

ATP is involved in TG-induced apoptotic cell death, PMA-differentiated THP-1 macrophages were incubated with TG in the presence or absence of ATP to assess cell viability. Treatment of THP-1 macrophages with ATP and TG resulted in a greater reduction in cell viability compared to treatment with TG only (Fig. 2A). Subsequently, to investigate whether extracellular ATP enhances apoptotic cell death in TG-treated macrophages, PARP cleavage was confirmed by western blot analysis. Extracellular ATP increased the levels of cleaved PARP in TG-treated macrophages (Fig. 2B). In addition, extracellular ATP also increased the activity of caspase-1 and 2 in TG-stimulated macrophages (Fig. 2C and 2D). To elucidate whether the increase in extracellular ATP was responsible for an increased TG-triggered apoptotic cell death, THP-1 macrophages were treated with TG in the presence of the ATP-depleting enzyme apyrase, which catalyzes the hydrolysis of ATP and ADP to AMP and inorganic phosphate. Cell viability was recovered (Fig. 2E) and the cleavage of PARP and apoptotic caspases (caspase-3, -7, -8, and -9) was reduced in the presence of apyrase in TG-treated THP-1 macrophages (Fig. 2F). Moreover, ATP depletion decreased the activity of caspase-1 and 2, which was increased in response to TG treatment (Fig. 2G and 2H). These results suggested that extracellular ATP is associated with TG-triggered macrophage death.

Activation of the pannexin-1 channel mediates TG-induced THP-1 macrophage cell death

Pannexin-1 channels are mechanosensitive conduits for ATP release in human cells (17). These channels are activated upon the cleavage of their C-terminus, and as they are activated, their cleavage fragments accumulate in the intracellular pool (18). To elucidate whether pannexin-1 is involved in macrophage cell death following TG treatment, THP-1 macrophages were treated with TG, and cytoplasmic pannexin-1 cleavage was measured using Western blot analysis. As a result, TG treatment increased cleaved pannexin-1 in a dose-dependent manner and pannexin-1 cleavage began to increase from 1.5 h after treatment (Fig. 3A).

When the pannexin-1 channel is activated, both ATP and small dye molecules can pass through it (18). An Et-Br uptake

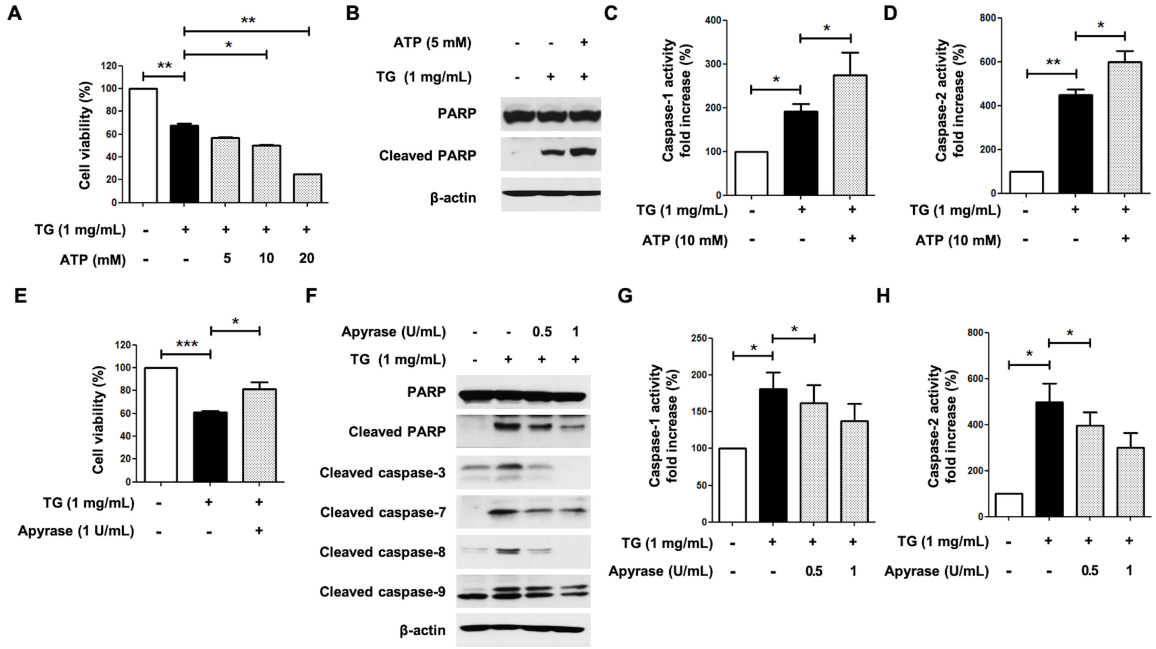

Fig. 2. An increase in extracellular ATP induces TG-triggered macrophage cell death. (A) THP-1 macrophages were incubated with TG in the presence of ATP for 24 h and the trypan blue exclusion assay was performed. The number of viable cells in THP-1 macrophages without TG treatment was set as 100%. (B) The cleaved form of PARP was detected by Western blotting. (C) THP-1 macrophages were incubated with TG in the presence of ATP for 24 h, after which caspase-1 activity was assessed. The absorbance of THP-1 macrophages without treatment TG was set as 100%. (D) THP-1 macrophages were incubated with TG in the presence of ATP for 24 h and caspase-2 activity was assessed. (E) THP-1 macrophages were incubated with TG in the presence of the ATP hydrolysis enzyme apyrase for 24 h, and viable cells were enumerated. The number of viable cells in THP-1 macrophages without TG treatment was set as 100%. (F) The cleaved form of PARP, and the cleavage of caspase-3, -7, -8, and -9 were detected by Western blotting. (G) THP-1 macrophages were incubated with TG in the presence of apyrase for 24 h, after which caspase-1 activity was assessed. The absorbance of THP-1 macrophages without TG treatment was set as 100%. (H) THP-1 macrophages were incubated with TG in the presence of apyrase for 24 h and caspase-2 activity was assessed. All data are expressed as the mean ± SEM of three independent experiments. P-values were determined with Student's t-test. *P < 0.05, **P < 0.01, *** P < 0.001.

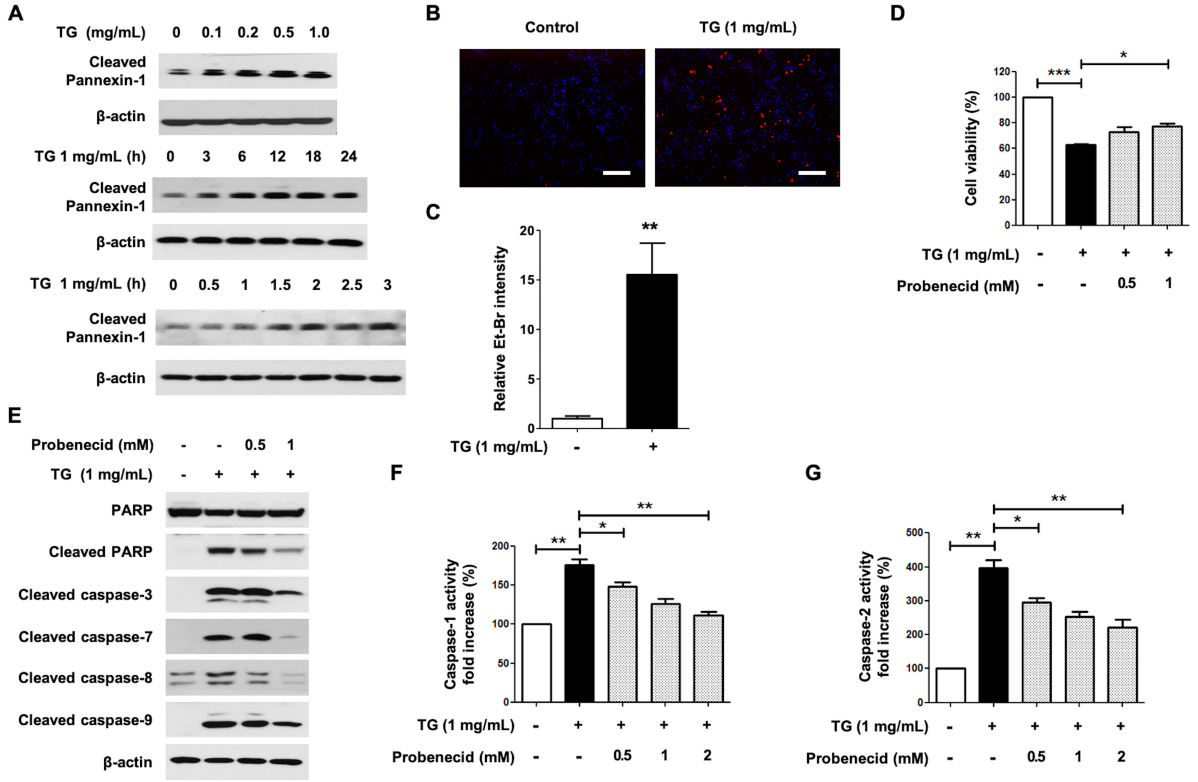

Fig. 3. TG-induced cell death involves activation of pannexin-1 channel in THP-1 macrophages. (A) THP-1 macrophages were incubated with the indicated concentration of TG for the indicated times. The cleaved form of pannexin-1 was detected by Western blotting. (B) THP-1 macrophages were incubated with or without TG for 24 h and an ethidium bromide uptake assay was performed to detect dye uptake by pannexin-1 channels (scale bars, 200 μm). (C) Relative ethidium bromide intensity measured. The fluorescence intensity of THP-1 macrophages without TG treatment was set as 1.0. (D) THP-1 macrophages were incubated with TG in the presence of the pannexin-1 channel inhibitor probenecid for 24 h, then viable cells were enumerated. The number of viable cells in THP-1 macrophages without TG treatment was set as 100%. (E) The cleaved form of PARP, and the cleavage of caspase-3, -7, -8, and -9 were detected by Western blotting. (F) THP-1 macrophages were incubated with TG in the presence of probenecid for 24 h, after which caspase-1 activity was assessed. The absorbance of THP-1 macrophages without TG treatment was set as 100%. (G) THP-1 macrophages were incubated with TG in the presence of probenecid for 24 h and caspase-2 activity was assessed. All data are expressed as the mean ± SEM of three independent experiments. P-values were determined with Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

assay was performed to evaluate the activation of the pannexin-1 channel after THP-1 macrophages were treated with TG. More fluorescent red dots were observed in TG-treated macrophages than those that were not (Fig. 3B and 3C). These results show that TG treatment increases pannexin-1 channel activity in THP-1 macrophages.

To determine whether the activity of pannexin-1 is involved in TG-induced macrophage death, TG-treated THP-1 macrophages were treated with probenecid, an inhibitor of pannexin-1. When the activity of pannexin-1 in TG-treated THP-1 macrophages was inhibited by probenecid, cell viability was recovered (Fig. 3D), and the cleavage of caspase-3, -7, -8, and -9, and PARP were decreased (Fig. 3E). The activity of caspase-1 and caspase-2 in TG-treated THP-1 macrophages was also reduced (Fig. 3F and 3G). Taken together, these results indicate that pannexin-1 channel activation mediates TG-triggered cell death in THP-1 macrophages.

TG-triggered cell death is mediated by ATP released by pannexin-1 channel activation

To test whether the activation of the pannexin-1 channel contributes to extracellular ATP-dependent caspase-1 and -2 activation and increases TG-induced cell death, THP-1 macrophages were incubated with TG in the presence of probenecid and ATP. The cell viability recovered upon the inhibition of the pannexin-1 channels was reduced again by the presence of the extracellular ATP in a dose-dependent manner (Fig. 4A). In addition, the cleavage of the caspases and PARP was increased again in response to the presence of ATP (Fig. 4B). The activity of caspase-1 and 2 decreased upon probenecid treatment was increased again in response to ATP treatment in a dose-dependent manner (Fig. 4C and 4D). Taken together, these results suggest that the

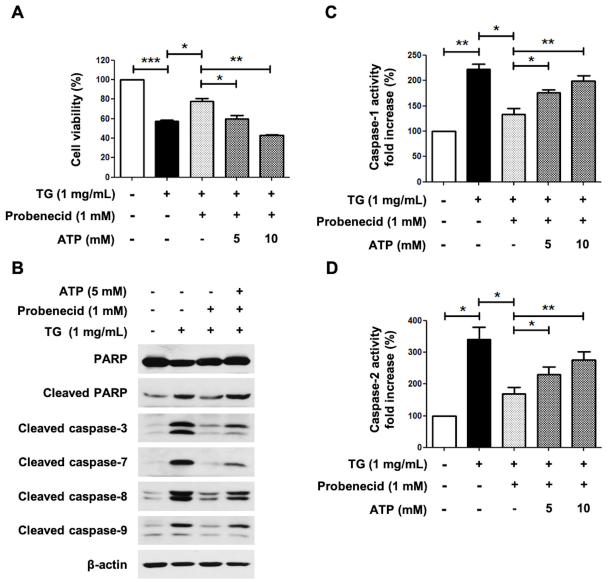

Fig. 4. TG-triggered cell death is mediated by ATP released via activated pannexin-1 channels. (A) THP-1 macrophages were incubated with TG in the presence of probenecid and ATP for 24 h and viable cells were enumerated. The number of viable cells in THP-1 macrophages without TG treatment was set as 100%. (B) The cleaved form of PARP, and the cleavage of caspase-3, -7, -8, and -9 were detected by Western blotting. (C) THP-1 macrophages were incubated with TG in the presence of probenecid and ATP for 24 h, after which caspase-1 activity was assessed. The absorbance of THP-1 macrophages without TG treatment was set as 100%. (D) THP-1 macrophages were incubated with TG in the presence of probenecid and ATP for 24 h then caspase-2 activity was assessed. All data are expressed as the mean ± SEM of three independent experiments. P-values were determined with Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

activation of the pannexin-1 channel mediates ATP-dependent TG-triggered cell death in THP-1 macrophages.

DISCUSSION

Macrophage cell death is an essential phenomenon in atherosclerosis, contributing to the development of plaques in advanced lesions (19). Since Oxi-LDL has been considered a key player in the development of atherosclerosis, much research has been directed at understanding the association between Oxi-LDL and macrophage death (20, 21). In contrast to Oxi-LDL, the role of TG in atherosclerosis has long been controversial. However, emerging evidence, including a recent large cohort study, suggested that elevated TG levels can be a causal risk factor for atherosclerosis (22). Thus, in the current study, we showed that i) TG increased extracellular ATP via pannexin-1, which in turn, ii) activates ATP-sensitive potassium channels, and that iii) there is an activation of the caspase cascade through increased potassium efflux. These results suggest that the TG-induced cell death of macrophages is mediated by pannexin-1 activation.

Pannexin-1 was identified as a plasma membrane hemichannel that releases ATP from the cytosol to the extracellular space during apoptosis (23). The released ATP can act as a "find-me" signal to recruit phagocytes and activate the initiation of inflammation, which can be an important contributor to the pathogenesis of atherosclerosis (23). We previously demonstrated that TG treatment enhanced the secretion of a pro-inflammatory cytokine, IL-1β, in THP-1 macrophages (6). Another function of increased extracellular ATP is the activation of ATP-sensitive potassium channels, subsequently leading to an increase in potassium efflux. Several investigations suggested that potassium channel activation contributes to the pathogenesis of atherosclerosis (24, 25). Ling and colleagues showed that the inhibition of ATP-sensitive potassium channels ameliorated atherosclerosis in $ApoE^{-/-}$ mouse models, directly elucidating the role of ATP-sensitive potassium channels in atherosclerosis (25). Moreover, they observed that the number of ATP-sensitive potassium channels in macrophages increased in atherosclerotic plaques, which may be explained by the present study. Here, we showed that TG-mediated macrophage cell death was dependent on the activation of ATP-sensitive potassium channels. It is possible that increased ATP-sensitive potassium channels in macrophages cause an increase in potassium efflux, leading to macrophage cell death. This may then potentiate the development of more vulnerable plaques.

In conclusion, our results provide a novel mechanism regarding how TG induces macrophage cell death. Since an enhanced level of macrophage death is considered a crucial phenomenon in plaque development in atherosclerosis, our results imply that TG-induced macrophages can create plaques and produce thrombi, resulting in clinical ischemic cardiomyopathy. Furthermore, our results support that TG can be a risk factor in the pathogenesis of atherosclerosis, and that control of TG levels can be a valuable therapeutic target for the disease.

MATERIALS AND METHODS

See supplementary information for Materials and Methods.

ACKNOWLEDGEMENTS

This research was supported by the Korea Nazarene University Research Grant 2020.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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