Author's response to reviews

Title: Cardiac thromboxane A2 receptor activation does not directly induce cardiomyocyte hypertrophy but does cause cell death that is prevented with gentamicin and 2-APB

Authors:

Chad D. Touchberry (cdtchbrr@memphis.edu)
Neerumpma Silswal (silswaln@umkc.edu)
Vladimir Tchikrizov (tchikrizov@gmail.com)
Chris Elmore (Christopherj.blough@gmail.com)
Shubra Srinivas (srinivass@umkc.edu)
Adil S. Akthar (asa7b2@mail.umkc.edu)
Hannah K. Swan (swanh@william.jewell.edu)
Lori A. Wetmore (wetmorel@william.jewell.edu)
Michael J. Wacker (wackerm@umkc.edu)

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Author's response to reviews: see over
We kindly thank Drs. Chiong and Kawabe for their time and helpful insight in the reviews. Their comments have strengthened the manuscript. We have revised the manuscript based on the reviews and have included responses to each comment below:

Reviewer #1
1. Authors described the TXA2 receptor mRNA in AVCMs, as well as the increase of calcium upon U46619 treatment. Because HL-1 cells were also used in this work, the presence of TXA2 receptor in these cells should be also evaluated. Control on U46619-induced calcium increase using TXA2 receptor antagonist is also desirable.

The reviewer raises a good point. We performed real-time RT-PCR on AVCM as well as HL1 cell RNA in the same runs and we have analyzed this data using both β-actin and GAPDH as reference genes. TXA2 receptor mRNA was expressed in HL-1 cells similar to primary cells. Comparing the expression between AVCMs and HL-1s, there was a 3.2 fold higher expression of TXA2R in primary cells compared to HL-1 cells using β-actin and 2.7 fold higher expression using GAPDH as the reference gene. We have included this data in the results section (p.12 line # 306-309) and discussion (p. 16 line 384). We also tested calcium responses in HL-1 cells during our initial series of experiments. We observed an increase in intracellular calcium after U46619 treatment using fura-2 ratiometric fluorescent imaging with the average relative fluorescent units being 0.51 ± 0.2 at baseline and increasing on average to 1.01 ± 0.16 at peak response to U46619 (n=14 cells). We have included this data in the results section (p. 12 line # 315-316) and discussion (p. 16 line # 386). Unfortunately, we did not test the TXA2 receptor antagonist, SQ29548, with this group of cells since we have previously inhibited U46619-induced calcium responses with SQ29548 (1) and Dogan et al. (2) have shown antagonism of U46619-induced calcium changes in cardiomyocytes by SQ29548 and Hoffmann et al. (3) has also shown that the TXA2 receptor antagonist SK&F95585 blocks U46619-induced cardiomyocyte calcium.

2. Hypertrophic genes expressions were only evaluated in AVCMs. Induce U46619 the expression of atrial natriuretic peptide, #-myosin heavy chain, and skeletal muscle actin in HL-1 cells?

We conducted hypertrophic gene expression experiments in which HL-1 cells were treated with U46619 similar to the AVCM protocol. We found that U46619 also did not induce a significant increase in expression of β-MHC (1.14 ± 0.07 fold), ANP (1.39 ± 0.31fold), or SkAct (1.8 ± 0.75 fold) (P>0.05; n=4). We have included this data in the results (p. 13 line # 332-335). These results support Fig. 2A that HL-1 cell size was not increased by U46619 and Fig. 3 that hypertrophic genes were not increased in primary cells by U46619 treatment.

3. Is cell death (MTT and trypan blue assay in HL-1 and cell shape in AVCMs) prevented by SQ29548, 2-APB and gentamicin?

We conducted manual cell counts of viable primary AVCMs with SQ29548, 2-APB, and gentamicin pretreatments for 3 experiments. We found that 10 μM U46619 reduced viable cells by -20.3 ± 1.7% compared to vehicle treatment; however, the reduction in viable cells by U46619 was prevented by pretreatment with SQ29548 (-1.4 ± 3.0%), gentamicin (-4.3 ± 6.2%), and 2-APB (-1.2 ± 1.9%) (P<0.05). These results match and support the TUNEL staining findings in Fig. 5. We have added these findings to the results section (p. 15 line # 359-362), as well as the discussion (p. 19 line # 449-450).

4. TUNEL assay is cannot clearly distinguish between apoptosis or necrosis. Did authors perform other assays such as LDH release, cytochrome C relocalization or caspase-3 activation?
We have previously performed LDH assays at 4-6 hours on HL-1 cells using 1 and 10 μM U46619 (n=3). Total maximum LDH release was determined by permeabilizing cells with 2% triton X. The % LDH release was less than 1% of total LDH for both 1 and 10 μM concentrations (0.8 ± 0.8% and 0.4 ± 0.7%, respectively). Typically an increase in LDH leaking from cells at this time point would indicate necrosis or oncosis. Therefore, U46619 did not induce immediate necrosis, and may have caused cell death by inducing apoptosis. This finding is supported by previous research that showed that U46619 did not induce LDH leakage in neonatal rat cardiomyocytes from 1-4 hours (2) and that activation of the TXA2 receptor induced apoptosis in cardiomyocytes (4,5) as well as endothelial cells (6), renal tubule cells (7), and immature thymocytes (8). Despite these findings, the reviewer raises a good point, and it is possible that the issue may be more complex than previously reported. It may be beneficial to fully examine if U46619 is inducing apoptosis, autophagy, necrosis, oncosis, or a combination of these events in an additional study using multiple assays, time points, sample numbers, and dosages. However, in this initial study, we are primarily concerned if TXA2 receptor activation induces cell death over hypertrophy and if cell death can be blocked by inhibitors of the IP3 pathway. We have revised our results and discussion in order to be consistent to refer to our findings simply as ‘cell death’ to ensure that we are not overstating our data. We have included our LDH assay data in the results (p. 14 p. 340-342) and have included additional discussion in the manuscript on this point raised by the reviewer (p. 18 line # 424-435).

Reviewer #2

1) The experimental studies have well been designed, and these data are supportive for their conclusion. However, the originality about the findings in this article is somewhat low. The negative finding that ‘Ca-mobilizing agonist’ did not induce cardio-hypertrophy is not surprising...

We thank the reviewer for the comment and insight. Our research group was actually somewhat surprised and intrigued that TXA2 receptor activation did not induce hypertrophy at any of the concentrations tested. Other research groups in addition to ours have shown that TXA2 receptor activation stimulates Gq, PLC, PKC, and IP3-induced release of calcium similar to the effects of angiotensin II, norepinephrine, phenylephrine, and endothelin-1 on the heart. All of these other hormones/agents have been well documented to induce cardiac hypertrophy. Therefore, there was a strong rationale to hypothesize that TXA2 receptor activation would also induce hypertrophy. We feel that it is a unique finding that this Gq-linked hormone/paracrine agent induced cell death over hypertrophy. We have added a small part to the discussion to highlight and clarify this point (p. 17 line #400-402, 411-414)

2) There are several reports that U46619 (dose 1-10uM) affect the several cardiac functions including arrhythmia (ref 16-19, 24 including the authors report). In this study, the detrimental effect of U46619 on cardiomyocytes, i.e. induction of cell death was too high. If so, most of the functional abnormality in the presence of U46619 is somewhat non-specific phenomenon. Indeed, U46619 did not occurred cell damage in neonatal rat myocytes, assessed by the LDH leakage (ref 19), while other TXA2 receptor agonist, IBOP induce apoptosis in adult rat cardiomyocytes (ref 28). Please explain the discrepancy about the effects of U46619, and also a consistency with the myocardial functional disorder and severe cytotoxicity.

These are good inquiries by the reviewer. If we understand the reviewer correctly, the first part of the inquiry is asking to explain the connection or to tie together the ‘detrimental’ (cell death) effects versus the previously documented ‘functional’ effects (calcium mobilization, arrhythmias, cardiomyopathy, etc.) by TXA2R activation and specific vs non-specific actions. As the reviewer indicated, we have previously shown that U46619-induced calcium entry in vitro and arrhythmias in vivo
in a previous study (1). The calcium changes and arrhythmias had a rapid onset (seconds and minutes, respectively) and were eliminated by pretreatment with the TXA2 receptor antagonist SQ29548. Now in this study we show that cell death occurred at 24 hours and the cell death and DNA fragmentation was also blocked by SQ29548 and IP3 inhibitors in vitro (which avoids other confounding factors). Based on this data, we would hypothesize that both effects were mediated by the TXA2 receptor and thus were specific to TXA2 receptor signaling. We would propose that when TXA2 is elevated in the heart, there is an increase in intracellular calcium in cardiomyocytes which transiently can induce changes in contractility as well as trigger arrhythmias. If the subject survives the initial arrhythmic event, we hypothesize that sustained increases in calcium or activation of cell death pathways caused by TXA2 receptor stimulation (hours to days) induces some cardiomyocytes to die. This death of some cardiomyocytes potentially induces the changes in ejection fraction and remodeling that has been observed in other studies we cited (5,9). Therefore, the type of effect observed is likely mediated by the time of exposure and assay endpoint. We have added a section with these comments to the discussion (p. 19-20 line # 460-474).

Another inquiry was to explain the discrepancy in that one previous study has shown that U46619 did not induce cell damage/death (3) vs. another study in which another TXA2 receptor agonist IBOP caused apoptosis (4). Hoffman et al. (3) only determined cytotoxicity via LDH activity after 1 or 4 hours of administration of U46619 to ensure that the U46619 did not cause immediate damage to cellular membranes. An increase in LDH activity (or increase in cell death) during the 1-4 hour time point would likely be a result of necrosis. Thus, the authors concluded that the U46619-induced increases in calcium were not due to the leakage of calcium via loss of membrane integrity of nonspecific destruction of membranes. If cell death/LDH leakage occurs at 24 h this is more likely due to apoptosis. Therefore, the Hoffman et al. (3) data is similar to our LDH data (See reviewer 1) and actually supports the findings by others suggesting that U46619 does not induce necrosis, but apoptosis. Hoffman et al. did not look at any cell death time points beyond 4 hours so it is difficult to further compare to our study or Shizukuda and Buttrick (4) that were done at 24 hours. Our results are similar to Shizukuda and Buttrick not only in the time point but also the increase in DNA fragmentation via TUNEL staining and that the cell death was inhibited by SQ29548. We thank the reviewer for pointing this out and we have included this comparison in the discussion (p. 18 line # 424-435).

References


