The Damaging Interplay between Extracellular RNA and Tumor-Necrosis-Factor-α in Cardiac Ischemia/Reperfusion Injury: Prevention of Cardiomyocyte Death and Heart Failure by RNase1

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Abstract

Background - Despite optimal therapy, the morbidity and mortality of patients presenting with an acute myocardial infarction (MI) remain significant. Here, we investigate extracellular RNA (eRNA) as a novel therapeutic targets for protecting the heart against the detrimental effect of acute ischemia/reperfusion (I/R) injury.

Methods and Results - We show that eRNA and tumor necrosis factor-α (TNF-α), released by the myocardium in response to I/R, mediate cardiomyocyte death. In patients subjected to acute global I/R during cardiac bypass surgery, significant elevations in plasma levels of eRNA and TNF-α were found. Moreover, following *in vivo* myocardial I/R in mice or I/R induced in the isolated Langendorff-perfused rat heart, both eRNA levels and cardiac injury markers were increased. Similarly, in cardiomyocytes subjected to hypoxia, eRNA was released resulting in TNF-α liberation through the activation of TNF-α converting enzyme (TACE). Conversely, TNF-α promoted further eRNA release especially under hypoxia, feeding a vicious cell damaging cycle during I/R with the massive production of oxygen radicals. The administration of RNase1 or TAPI (a TACE-inhibitor) prevented cell death and reduced myocardial infarction. Likewise, RNase1 significantly reduced I/R-mediated energy exhaustion, opening of the mitochondrial permeability transition pore as well as oxidative damage in cardiomyocytes.

Conclusions - RNase1 and TACE-inhibition provide novel therapeutic strategies for counteracting the adverse eRNA-TNF-α interplay which occurs during I/R, thereby preventing cell death, limiting myocardial infarct size and preserving cardiac function. This newly discovered fundamental pathogenic mechanism of I/R injury is likely to operate in other organs and tissues as well.

Introduction

Cardiomyocyte death occurring during acute myocardial infarction has a significant impact on the quality of life and survival of patients suffering from coronary artery disease, the most eminent single cause of death worldwide[1]. Severe myocardial ischemia secondary to a thrombotic coronary occlusion at the site of a ruptured atheromatous plaque results in extensive cardiomyocyte death involving most of the area at risk unless coronary blood flow is rapidly restored by pharmacological or mechanical interventions[2]. However, although early reperfusion salvages viable myocardium and limits infarct size and markedly improves the prognosis of patients with acute myocardial infarction, its beneficial effect is limited by the occurrence of additional cardiomyocyte death during the initial minutes of reflow, a phenomenon described as lethal ischemia/reperfusion (I/R) injury[3]. This paradoxical phenomenon, has largely been studied in experimental animal models and is known to be caused by a network of mechanisms causing Ca^{2+}-overload[4], mitochondrial permeabilization[5, 6] or ATP-dependent hypercontracture[7, 8] that may limit myocardial salvage achieved by reperfusion[9]. Experimental studies have convincingly demonstrated that infarct size can be markedly reduced by therapeutic interventions applied at the time of reperfusion, such as ischemic postconditioning[10] and diverse pharmacological interventions aimed to improve intracellular calcium handling and reduce proteolytic activation. Moreover, heart protection was experimentally achieved by reduction of hypercontracture and mitochondrial permeability transition[11], modulation of Na^+/H^+ ion exchanger[12] or application of particulate guanylyl-cyclase agonist[13]: However, none of these approaches have been introduced into clinical practice. Nevertheless, application of non-invasive intermittent limb I/R (designated remote ischemic preconditioning) either applied prior to heart operations[14] or applied by a paramedic to patients with ST-Segment elevation myocardial infarction (STEMI)[15] upon primary percutaneous coronary intervention (PPCI) have recently been found to be beneficial for the outcome of cardiac ischemic damage.

Experimental studies thus far have not taken into account that factors released from the damaged cardiac tissue itself may have detrimental effects on reperfused cardiomyocytes and contribute to lethal reperfusion injury and final infarct size. Although it is well known that disrupted cells may release cytosolic proteins during reperfusion, such as creatine kinase or cardiac troponins, these and other factors have only been used for diagnostic evaluation[16, 17]. Another component that is detectable in the extracellular space during tissue hypoxia or cell damage are ribonucleic acids (RNA), which are released by damaged cells and designated as extracellular RNA (eRNA)[18]. The majority of eRNA consists of ribosomal RNA, which binds to different basic proteins in blood plasma or on cell surfaces, and thereby promotes a variety of extracellular functions, particularly related to vascular diseases[19, 20]. These include activation of blood coagulation / thrombosis, release of TNF-α and other cytokines as well as promotion of inflammatory processes, such as leukocyte trafficking[21]. Here, we demonstrate an obligate role for eRNA and TNF-α as early alarm signals in cardiac tissue damage that determine the cell fate during cardiac I/R. Consequently, in the current study, we demonstrate that the administration of ribonuclease1 (RNase1) to inhibit eRNA or an antagonist of TNF-α-converting enzyme successfully reduced pathological parameters that are characteristic of I/R injury and substantially improved the early post-ischemic functional myocardial recovery.

Material and Methods

Extracellular RNA (eRNA) quantification

eRNA was quantified in plasma obtained from eight patients undergoing cardiac surgery (who all gave written informed consent, approved by the Ethics Committee, Medical Faculty, Justus-Liebig-University, Giessen; file number 166.11), in mice plasma, in the perfusate obtained from the isolated Langendorff heart as well as in conditioned medium from rat cardiomyocytes using the Master Pure[TM] RNA Purification kit (Epicentre Biotechnologies). Total RNA concentration was quantified with NanoDrop ND-2000 (peqLab Biotechnologie GmbH). RNase1 activity was quantified in human plasma or perfusate by an enzymatic assay as described[22].

Treatment with RNase 1 or RNase-inhibitor (RI) after induction of ischemia / reperfusion (I/R) in mice.

The established *in vivo* experimentation was exactly carried out as previously described[23]. Animals were treated with a continuously treated (started three days before surgery) with RNase1 (Fermentas®, 100 µg/mouse) or RNase Inhibitor (RiboLock, Fermentas®, 80 U/mouse) via a subcutaneously implanted miniature infusion Alzet® osmotic pump (model 1004). Quantification of the infarcted area and *in vivo* assessments of cardiac functions were done as described[23]. All animal experiments and study protocols were approved by local authorities of the RWTH Aachen, complying with Romanian and German animal protection laws. Experiments involving TNF-deficient mice (TNF-KO) were approved by the Health Science Faculty Animal Ethics Committee, University of Cape Town. Animals were housed and treated in accordance with the Guide for Care and Use of Laboratory Animals (Eighth Edition), published by the US National Institute of Health Publications. TNF-KO from a C57BL/6 genetic background, were a generous gift from Dr. Jacobs and Prof. Ryffel (Department of Immunology, University of Cape Town)[24].

Langendorff heart perfusion system

Wistar rats (10-12 weeks old; weighing 225–300 g) were specific pathogen free, and kept at the animal care facility, Justus-Liebig-University, Giessen, with free access to food and water, conforming to the Guide for the Care and Use of Laboratory Animals, National Institute of Health (NIH publication no. 85-23, revised 1996). Experiments were performed on isolated hearts as previously described[25]; experimental conditions are outlined in the corresponding legends to Figures.

Quantification of cell death

Lactate dehydrogenase (LDH) activity in the Langendorff-perfusate was spectro-photometrically measured during the entire reperfusion period of 120 min. Thereafter, hearts were cut into four slices and incubated at 37°C for 10 min in 1% triphenyltetrazolium chloride (pH 7.4) and imaged under white light to outline the area of necrosis[26]. LDH release into the cardiomyocyte-conditioned medium was assessed by a detection kit (Roche Diagnostics)[27].

Isolation of adult mouse and rat cardiomyocytes

Ventricular heart muscle cells were isolated from 12-weeks old rats or mice as previously described[28, 29]. Following hypoxia, cells were loaded with 0.04% trypan blue and cell viability was immediately analyzed using a light microscope at 40× magnification. The number of viable (unstained) and nonviable (blue stained) cells was recorded as previously described[28].

Isolation of extracellular RNA and DNA for cardiomyocyte treatment

Following washing of confluent cultures of smooth muscle cells with PBS, total RNA was isolated with GenElute Mammalian Genomica RNA Miniprep Kit (Sigma-Aldrich). RNA was hydrolysed by incubation with RNase1 (Fermentas®) for 1h at 37°C. Hydrolysed RNA was always used at the same concentration as cellular RNA. Total RNA concentration was

quantified with the NanoDrop ND-2000 (peqLab Biotechnologie GmbH). The quality of total RNA and hydrolysed RNA was determined with the 2100 Bioanalyzer using "Eukaryote total RNA Nano Assay" (Agilent Technologies). All chips were done in duplicates. DNA was isolated with GenElute Mammalian Genomica DNA Miniprep Kit (Sigma-Aldrich) from confluent cultures of smooth muscle cells. Quality of total DNA was confirmed by electrophoresis on a 1% agarose gel followed by ethidium bromide staining.

Induction of mitochondrial membrane permeabilization

The preparation of rat cardiomyocytes and experimental procedures were performed as previously described[4].

ROS immunolabeling and fluorescent microscopy

Hearts from the different experimental groups were removed from the Langendorff system, weighed and rapidly frozen in liquid nitrogen. *In situ* reactive oxygen species (ROS) were determined by labelling with dihydroethidium as described[30].

Quantification of TNF-α

Langendorff heart-perfusates or cardiomyocyte conditioned media were collected and filtered through 0.2 μm filter to remove any residual debris with subsequent quantification of TNF-α **(for details see supplementary information)**.

RNA isolation and Quantitative Real Time PCR analysis (qRT-PCR)

DNA-free total RNA was extracted from homogenised heart tissue or from cardiomyocytes treated under different conditions and lysed using *TRIzol® Reagent (Invitrogen, USA)*. Standard procedures were used for qRT-PCR **(for details see supplementary information)**.

Western blot analysis

Homogenised heart tissue or cardiomyocytes treated under different conditions were lysed using RIPA buffer and prepared for denaturing SDS gel-electrophoresis and western blotting as previously described [21].

Statistics

Data were analyzed by unpaired Student's t test or One-way ANOVA analysis of variance followed by Tukey's, Dunnett's or Bonferroni's multiple comparisons test was performed, when appropriate, to determine statistical significance of the data differences using GraphPad Prism version 6.00 **(for details see supplementary information)**. Results were considered as significantly different at $p < 0.05$.

Results

Association between extracellular RNA and TNF-α in myocardial ischemia/reperfusion injury during cardiac surgery.

Upon tissue damage or vascular injury, significant amounts of extracellular RNA (eRNA) (composed predominantly of 18S and 28S ribosomal RNA) are released that promote procoagulant and proinflammatory processes, as was observed in our earlier studies[18, 22, 31]. To characterize the pathogenic situation of cardiac ischemia/reperfusion (I/R) injury in this respect, 14 cardiac patients **(Supplementary Table 1)** were analysed for eRNA and tumor necrosis factor-α at different time points during surgery: Blood samples were taken from the radial artery, directly before and after aortic clamping as well as from the coronary sinus. **Figure 1A** demonstrates a massive increase by 10 to 20 fold of eRNA (predominantly intact 18S/28S rRNA) **(Figure 1B)** and a significant elevation of TNF-α (>7-fold) **(Figure 1C)** particularly in coronary sinus blood after aortic clamping. This indicates that ischemic, albeit cardioplegia-protected, human myocardium is a major source of eRNA during cardiac surgery which may, in concert with TNF-α, contribute to the pathogensis of I/R injury and myocardial infarction.

eRNA promotes I/R injury and myocardial infarction.

In order to prove the release of eRNA upon ischemic heart disease, a myocardial infarction model in mice and the isolated rat Langendorff heart model, exposed to I/R injury, were investigated. Following coronary artery ligation in mice for 60 min, eRNA levels were significantly elevated during the subsequent 120 min reperfusion phase **(Figure 2A)**. In order to delineate the cellular origin of eRNA, RT-PCR analysis was carried out to amplify cell-specific mRNA that was found associated with the released 18S/28S rRNA **(Figure 2B, insert)**. Results indicate that eRNA is derived mainly from cardiomyocytes (expressing SERCA[32]) and to a much lesser degree from smooth muscle cells[33] (expressing α-smooth

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muscle actin) and myofibroblasts (expressing bHLH transcription factor - *Tcf21*[34]) **(Figure 2B)**.

After excision and perfusion equilibration of isolated rat hearts on the Langendorff apparatus, blood-free perfusion was arrested for 45 min to achieve global ischemia and thereafter, reperfusion was resumed for 120 min. At the start of the reperfusion phase, a significant increase in eluted eRNA was observed **(Figure 2C)**, together with other markers of myocardial injury such as lactate dehydrogenase (LDH), cardiac troponins and creatine kinase[35] (data not shown). In contrast to the latter parameters, a second washout peak of eRNA during reperfusion between 15 and 60 min was noted that related to more than 60% of the totally recovered eRNA. Analysis of eRNA collected from the entire 120 min reperfusion phase revealed a significant portion of microparticle-associated eRNA **(Figure 2C, insert)**.

To further delineate these findings in a cell culture model, a significant increase in the accumulation of eRNA in the supernatants of isolated cardiomyocytes, exposed to hypoxia for 1h, as compared to 3h of normoxia, was observed **(Figure 2D)**. Moreover, exposure of cells towards TNF-α significantly enhanced the eRNA release, both under normoxic and hypoxic conditions.

Hypoxia and eRNA promote cardiac I/R-induced TNF-α release.

To define the conditions of I/R injury under which TNF-α release occurs, *in vivo*, *ex vivo* and *in vitro* experiments were performed. While in the murine heart ligation model under constant perfusion no significant TNF-α liberation was observed in the animal circulation **(Figure 3A)** as well as in heart tissue **(Figure 3B)**, exposure to ischemia for 60 min, followed by various time intervals of reperfusion (0, 2 and 120 min) all resulted in massive increase in TNF-α release with I/R of 60 min/2 min showing the highest value (>20 fold) **(Figure 3A)**. These conditions were also associated with a dramatic appearance of TNF-α in heart tissue of these animals **(Figure 3B)**. While the administration of RNase1 resulted in a drastic reduction of

TNF-α release and tissue deposition, the application of an RNase inhibitor (RI) had no significant influence on the eRNA/hypoxia-induced liberation of TNF-α.

Upon I/R exposure in isolated rat hearts, significantly increased levels of TNF-α were detectable in the perfusate during the initial 15 min reperfusion phase in accordance with a report in which the gene for this cytokine was found to be transcribed during this I/R phase[36]. Treatment with RNase1 reduced the level of TNF-α almost to background values (**Figure 3C**), and after 120 min reperfusion, TNF-α mRNA was significantly decreased in the RNase1 treatment group (**Supplementary Figure 1A**). In contrast, administration of RI had neither influence on liberation of TNF-α nor on its mRNA expression when compared to the I/R group.

In previous studies, we have demonstrated that exposure of monocytes to eRNA resulted in the induction of TNF-α release, which largely relied on the sheddase TNF-α-converting enzyme (TACE/ADAM17)[21]. Likewise, upon stimulation of cardiomyocytes by eRNA under normoxic conditions, a significantly enhanced release of TNF-α was noted, while in the presence of RNase1, TNF-α shedding was minimal. Moreover, even under control conditions, RNase1 kept the level of TNF-α to a minimum (**Supplementary Figure 1B**). eRNA-induced TNF-α liberation was confirmed by data from a cytokine profile array, showing a significant increase of TNF-α under conditions of normoxia and hypoxia (**Supplementary Figure 1C**). Moreover, upon incubation of cardiomyocytes with eRNA for 1h under hypoxia, a significantly elevated TNF-α release was noted that was totally prevented by RNase1 treatment (**Figure 3D**). RNase1 alone was also effective under hypoxia as significantly reducing the level of released TNF-α (**Figure 3D**).

Production of reactive oxygen species (ROS) during I/R injury and prevention by RNase1.

The production of ROS during ischemic heart disease is a causally-related hall-mark in the pathogenesis of myocardial infarction, playing a significant role in damaging the heart during I/R[37, 38]. Upon exposure of mice to 60 min regional ischemia (by coronary artery ligation), followed by different time intervals of reperfusion, inspection of heart tissue by confocal microscopy revealed a markedly disturbed cardiomyocyte structure, accompanied by a significant elevation of ROS, which were found at intra-nuclear sites overlapping with the DAPI stain for condensed DNA, both in **(Figure 4A)**. The same holds true when tissue samples from the isolated Langendorff heart model, under conditions of 45 min ischemia and various reperfusion intervals, were inspected **(Figure 4B)**. In contrast, in RNase1-treated animals/isolated hearts, not only was the tissue architecture greatly preserved, but the fluorescence intensity of ROS was low, similar to control sections, not exposed to I/R. Cardiac tissue content of ROS (209 ± 11 AU/μm^2) in I/R hearts was reduced upon RNase1 treatment to 70 ± 12 AU/μm^2 (P<0.001). As expected, RI treatment did not prevent tissue destruction, and ROS accumulation was discernible as in the non-treated I/R injury groups.

To further substantiate the influence of anti-oxidant enzymes in cardiomyocytes on ROS production, RT-PCR analysis of peroxiredoxin 3 and isoforms of superoxide dismutase (SOD), representing cytoplasmic SOD1, mitochondrial SOD2 and extracellular SOD3, was performed in the Langendorff heart model as well as in isolated cardiomyocytes, exposed to the ischemia-related protocols. While low mRNA levels of these antioxidant enzymes were found in the isolated heart after 120 min reperfusion only, administration of RNase1 in isolated hearts submitted to I/R resulted in significantly elevated mRNA levels of these enzymes, particularly peroxiredoxin 3 and SOD 2 **(Supplementary Figure 2A)**. Likewise, upon exposure of isolated cardiomyocytes towards eRNA or TNF-α, respectively, for 3h under normoxia, significantly reduced mRNA levels of the indicated antioxidant enzymes were found **(Supplementary Figure 2B)**, whereas RNase1 application fully restored their

expression at normal values. The administration of the TACE-inhibitor TAPI in isolated cardiomyocytes led to a superimposable beneficial restoration of mRNA expression of the antioxidant enzymes as well (**Supplementary Figure 2B**).

RNase1 protects against cardiac I/R injury and reduces infarct size.

Following induction of acute cardiac I/R injury in the mouse model, infarct size expressed as a percentage of the area of risk was smaller in hearts administered RNase1 when compared to the application of buffer alone or RI, respectively (**Figure 5A**), indicative of the causal contribution of eRNA to cardiac tissue damage. Likewise, vehicle-treated mice after 14 days of reperfusion developed myocardial infarcts comparable to the RI-treated group ($16 \pm 3\%$ vs. $15 \pm 2\%$), whereas the RNase1 treatment group ($3 \pm 1\%$) was protected against infarct development (**Figure 5B**). Intra-ventricular measurements revealed a significant decrease in left ventricular developed pressure, contraction and relaxation in the control and RI-treated groups, whereas the RNase1-treated group exhibited preserved heart function comparable with sham or uninjured mice (**Supplementary Figure 3A-C**).

To characterize the detrimental effects of RNase1 upon physiological parameters in the Langendorff heart model under I/R conditions, three groups receiving increasing concentrations of RNase1 as well as one RI-group were studied. The drugs were administered together with the perfusion buffer prior to the induction of 45 min ischemia (**Supplementary Figure 4A**). Measurements of left ventricular end-diastolic pressure (LVEDP, a measure of the magnitude of ischemic rigor contracture) in the RI-group (34 ± 5 mmHg) revealed a similar or even higher value when compared to non-treated I/R hearts (26 ± 4 mmHg), whereas RNase1 in a concentration-dependent manner significantly reduced the adverse outcome of I/R (11 ± 3 mmHg at 10 µg/ml) and was effective over the entire reperfusion phase (**Supplementary Figure 4B**). Similarly, cardiomyocyte hypercontracture (a measure of I/R-mediated injury) was significantly reduced in the RNase1-treated groups (30 ± 4 mmHg at

10 µg/ml), while RI-administration (104 ± 8 mmHg) resulted in an even worse (although not significant) effect when compared to the untreated I/R group (66 ± 9 mmHg) **(Supplementary Figure 4C)**.

Furthermore, only minor quantities of LDH were detectable in all perfusate fractions in the RNase1 treatment groups, indicating markedly reduced necrotic cell death, whereas the typical early release of LDH following reperfusion was documented in the control and the RI-treated groups **(Figure 5C)**. The cumulative LDH release was markedly reduced in the RNase1 group (44 ± 7 UI/g tissue, *vs.* 127 ± 32 UI/g tissue in I/R group) **(Supplementary Figure 4D)**. Most importantly, RNase1-administration substantially decreased infarct size in the isolated heart (23% *vs.* 66 ± 5%, P<0.001) **(Figure 5D)**, while the application of RI did had no effect on these parameters when compared to I/R injury alone. Likewise, in the RNase1 treatment group, contractile recovery (expressed as left ventricular developed pressure, LVdevP) was significantly improved (73 ± 13% of basal values, *vs.* 38 ± 12% of basal values in I/R group) after 120 min reperfusion **(Supplementary Figure 4E)**.

Cytoprotective functions of RNase1.

While under normoxia, eRNA, RNase1, RI or DNA did not influence basal LDH release in cardiomyocytes, a substantial increase of LDH release was observed upon exposure of cells towards 1h hypoxia **(Figure 5E)**. Further addition of eRNA, but not hydrolyzed RNA, DNA or RI, respectively, resulted in significantly increased LDH release that was almost completely abolished by treatment with RNase1, independent of the presence of exogenous eRNA. Moreover, TNF-α markedly promoted LDH release, particularly under conditions of hypoxia **(Figure 5F)**, similar to the activity of eRNA **(see Figure 5E)**. Simultaneous addition of TNF-α and eRNA did not further enhance LDH release. Conversely, TAPI significantly suppressed eRNA-induced LDH release **(Figure 5F)**, but not to the same extent as it was demonstrated for RNase1-treatment in the hypoxia-induced LDH release **(see Figure 5E)**.

Ischemia in the isolated rat heart induced a rapid exhaustion of phosphocreatine as a measure of energy depletion with only partial recovery in the control I/R group (45 ± 5%) after 15 min of reperfusion, whereas in the RNase1 treatment group intracellular phosphocreatine remained elevated at a significantly higher level (73 ± 5%, P=0.032) and promoted substantial heart recovery (**Supplementary Figure 5A**). Since dysregulated opening of the mitochondrial permeability transition pore (mPTP) provokes mitochondrial dysfunction, which is associated with uncoupled oxidative phosphorylation and ATP hydrolysis, cardiomyocyte hypercontracture may ultimately lead to cell death[4, 39-41]. Exposure of cardiomyocytes towards eRNA significantly reduced the time of contracture related to mPTP opening (**Supplementary Figure 5B**) as assessed by the fluorescent dye tetramethyl-rhodamine-ethylester, which is readily sequestered by healthy mitochondria. In contrast, RNase1 abolished this effect as reflected by a value that was indistinguishable from the control. Furthermore, TNF-α promoted mPTP opening and induced cardiomyocyte hypercontracture, which became significant at a concentration of TNF-α at 50 ng/ml; anti-TNF-α reversed this effect (**Supplementary Figure 5C**). As an established protective agent, cyclosporin-A markedly prolonged the time to cardiomyocyte contracture, as expected.

Prevention of cardiomyocyte death by inhibition of TACE/ADAM17.

To specify the mechanism underlying eRNA-induced liberation of TNF-α and the subsequent adverse interplay of both components, the role of the metalloproteinase TACE/ADAM17 was explored in the established *ex vivo* and *in vitro* models. In the isolated rat heart, TNF-α release (maximal peak, 85 ± 10 pg/min *vs.* pre-ischemic values 12 ± 5 pg/min) during the initial reperfusion phase was significantly reduced upon treatment with TAPI (22 ± 8 pg/min) (**Figure 6A**). Likewise, in isolated cardiomyocytes, eRNA-induced TNF-α release was significantly prevented in the presence of the metalloproteinase inhibitors GM6001 or TACE-

specific TAPI **(Figure 6B)**. Furthermore, since activation of the NF-κB pathway was considered to be related to eRNA activity as well, TNF-α liberation was also blocked by the IκB phosphorylation inhibitor Bay11-708210 (Bay11). Neutralising antibodies against TNF-α completely blocked the TNF-α release mediated in the absence or presence of eRNA **(Figure 6B)**. These findings were corroborated by the observation that TAPI-treatment of the isolated rat heart during an interval of 30 min prior to the ischemic phase significantly decreased LDH release in comparison to the untreated I/R group **(Figure 6C)**. Furthermore, as compared to isolated wild-type cardiomyocytes, in TNF-α knockout cells, cell viability upon exposure to hypoxia decreased in a similar manner, but was not further reduced in the presence of eRNA **(Figure 6D)**, indicating the lack of TNF-α liberation due to the deficiency of the cytokine.

Using confocal microscopy to inspect I/R-exposed heart tissue as demonstrated before (see Fig. 4), administration of TAPI lead to the preservation of the cardiac tissue architecture, and fluorescence intensity of ROS was low as in the non-I/R group **(Supplementary Figure 6A)**. ROS content (211 ± 17 AU/µm^2) in I/R hearts was reduced upon TAPI-treatment to 91 ± 10 AU/µm^2 **(Supplementary Figure 6B)**, indicative of a similar protective role of this TACE-inhibitor as documented for RNase1.

The eRNA -TNF-α interplay promotes cardiac I/R-induced inflammation.

Upon ischemia in the isolated rat heart, the expression of the pro-inflammatory cytokine MCP-1 as well as iNOS was significantly increased, whereas treatment with RNase1 but not with RI prevented this effect **(Figure 6E)**. Likewise, stimulation of isolated cardiomyocytes with eRNA or TNF-α markedly increased the expression of MCP-1 and iNOS **(Figure 6F)**. In contrast, administration of RNase1 or TAPI, respectively, prevented the increase in mRNA levels of these inflammatory factors. Finally, Western blot analysis revealed that I/R injury in the isolated rat heart induced a significant decrease in IκB-α protein production, whereas

RNase1 treatment resulted in retaining the inactive NF-κB dimer by maintaining IκB-α in the non-phosphorylated state. Together, these data document the damaging role of eRNA and TNF-α in ischemia-related cardiac injury in different experimental models and propose a substantial protective function for RNase1 and TAPI.

Discussion

In the present study, novel mechanistic insights into the process of cardiac I/R injury that are governed by the endogenous extracellular RNA/RNase system were obtained. Moreover, the interplay between eRNA-dependent and TNF-α-related pathomechanisms characterized in this study may not be limited to ischemic heart disease but may be applied generally to ischemia-mediated damage of other organs or tissues as well, such that interventions with RNase1 or TACE-inhibitors would definitely provide new regimens for cytoprotection in general.

The rationale for studying the role of eRNA and TNF-α in ischemic heart disease was based upon our previous findings that these poly-anionic compounds promoted arterial thrombosis[18], induced the release of cytokines (including TNF-α), and served to elevate vascular permeability and edema formation *in vivo*[42]. Collectively, these functional activities of eRNA were largely inhibited by RNase1, which thereby plays a prominent role as a vessel- and tissue-protective agent[43]. Moreover, in cardiac patients undergoing cardiac bypass surgery, in which the heart is subjected to IRI, significantly elevated levels of eRNA (>20 fold) and TNF-α (>7-fold) were found in the present study, especially in coronary sinus blood before unclamping as compared to peripheral arterial blood. These data indicate massive release of these components particularly during an ischemic phase and are in accordance with earlier findings demonstrating elevated levels of TNF-α and IL-6 in the coronary sinus blood, suggestive for these authors to conclude that the lungs may consume rather than release proinflammatory cytokines in the early phase of cardiac reperfusion[44].

In fact, the eRNA - TNF-α relation in the coronary sinus may play an important role in the inflammatory response after aortic unclamping, not only because it may directly induce symptoms, but also because it may trigger the release of other cytokines, such as IL-6, IL-8 or IL-10[44-46]. In other reports, inflammatory responses were found to be the primary cause of microvascular incompetence in I/R injury, and global myocardial ischemia during aortic

cross-clamping seems to be one of the crucial pathogenetic factors in cardiac cytokine release and the "postperfusion syndrome" upon cardiopulmonary bypass[45, 46]. Together, these findings prompted us to further unravel the possible molecular interplay between eRNA and TNF-α under conditions of cardiac I/R injury in various experimental models.

In fact, acutely elevated levels of eRNA, both free and in association with microparticles, were found upon onset of reperfusion in the isolated rat heart model as well as in hypoxia-exposed cardiomyocytes in accordance with a recent report demonstrating the release of tissue factor-bearing microparticles from cardiomoycytes under inflammatory conditions[47]. Likewise, in acute models of vessel stenosis or chronic models of atherosclerosis in mice, we observed increased plasma and tissue concentrations of eRNA[33]. In the Langendorff heart model, the initial reperfusion-dependent washout of eRNA together with cardiomyocyte-specific markers such as creatine kinase or troponins was followed by a second peak of eRNA, whereby the vast majority of this 18S/28S rRNA material was derived from cardiomyocytes. Thus, unlike creatine kinase, troponins or LDH, eRNA remains to be released into the perfusate over an appreciable period of time where it can directly or indirectly affect cellular functions.

Since in isolated cardiomyocytes the combined action of TNF-α and hypoxia provoked eRNA release as well, we propose a reciprocal induction between eRNA and TNF-α that is particularly relevant under ischemic conditions. In fact, in the *in vivo* as well as in the *ex vivo* cardiac I/R injury model, substantial amounts of TNF-α were released and became deposited in cardiac tissue, whereas these TNF-α values remained almost at control level in the presence of RNase1. Collectively, these findings support our contention of an eRNA-promoted liberation of TNF-α and its reciprocal amplification in a positive feedback mechanism, particularly under conditions of I/R injury, with the result of generating appreciable amounts of the cytokine.

Since eRNA, in association with inflammatory processes, may provoke the release of TNF-α via a proteolytic shedding reaction, involving the metalloproteinase TACE[21], we tested whether this mechanism would apply to the setting of cardiac I/R injury as well. Indeed, TAPI not only prevented the generation of TNF-α in the *ex vivo* isolated heart model or in cardiomyocyte cultures, but also reduced cell death in these systems by blocking LDH-release and reducing the expression of damaging iNOS or MCP-1. In fact, the influence of TAPI on the outcome of these processes, all contributing to cardiac I/R injury and organ damage, was qualitatively superimposable with the outcome of RNase1 administration, indicative for the fact that a direct eRNA – TNF-α interplay is operative as pathological trigger.

Another group of major inducers of cellular damage under conditions of cardiac I/R injury are ROS, and their prominent contribution to organ damage is without doubt. Yet, the causal molecular relations that would lead to massive ROS generation under pathological conditions are incompletely understood. Here, we present strong evidence, both in the *in vivo* and *ex vivo* experimental model, that the elevation of ROS in plasma or perfusate as well as in association with the damaged myocardium was substantially reduced or prevented by RNase1 as well as by TAPI. Thus, it is concluded that the proven eRNA-TNF-α interplay provides a major stimulus for ROS production and deposition. Mechanistically, in combination with hypoxia/ischemia, eRNA provoked a marked reduction in the expression of antioxidant enzymes, indicative of the fact that such endogenous protective factors become down-regulated under stress conditions. Both, RNase1 or TAPI could reverse this effect by inducing the expression of such enzymes like peroxiredoxin 3 or the three isoforms of SOD in the Langendorff heart model or in isolated cardiomyocytes, respectively. In addition, RNase1 or TAPI reduced the expression of iNOS and MCP-1, factors which are known to be involved in mediating cell-damaging signals in cardiomyocytes. Together, these data strongly imply an

eRNA - TNFα interplay that appears to feed a vicious circle, culminating in ROS production and cardiomyocyte death.

The adverse outcome during I/R in the isolated rat heart as well as under hypoxic conditions in cardiomyocytes in response to eRNA in concert with TNF-α was also reflected by their negative influence on physiological cell parameters, including left ventricular-developed pressure, cardiomyocyte hypercontracture and mPTP opening. Here, administration of RNase1 resulted in markedly reduced contractility and left ventricular end-diastolic pressure (as a measure of the magnitude of rigor contracture) of the heart, as well as in very low levels of LDH in the perfusate as a general marker for necrotic cell death. Together with the massive reduction of ROS in cardiomyocytes as well as the preservation of tissue architecture, the multitude of physiological parameters that were protected by application of RNase1provide compelling evidence for its causative relation to tissue protection. Although it is tempting to assume that the beneficial effects of RNase1 treatment may depend on certain RNA degradation products such as adenosine[48, 49], we found no evidence for this relation. Likewise, 8-sulfophenyltheophylline (8SPT) did not influence the infarct size-reducing effect of RNase1, and 8SPT alone did not affect the control infarct size (**Supplementary Figure 7**).

Also, it should not be ignored that low doses of TNF-α may contribute to ischemic preconditioning protection by a direct effect on mitochondria[36,37]: In contrast to intervention with antibodies against TNF-α that would capture the entire pool of the cytokine and may induce adverse effects[38], the treatments with RNase1 and TAPI in this study prevented excessive TNF-α production, but left sufficient amounts of the cytokine, altogether supporting a protective outcome.

Finally, the overall measure of organ damage in cardiac I/R injury is ultimately quantitated by the degree of cell death and the respective infarct size. Both, RNase1 as well as TAPI significantly prevented LDH-release, albeit to a different degree. Here, RNase1 was superior to TAPI, particularly under hypoxic conditions, indicative for the fact that eRNA

indeed serves as initial upstream inducer of TNF-α liberation. Moreover, quantitation of infarct size in the acute or chronic *in vivo* disease model or the *ex vivo* Langendorff system revealed a massive protective effect in tissue recovery upon RNase1 administration. Thus, RNase1 as well as TAPI appear to be evidenced-based new protective drugs to limit cardiac infarct size, dependent on the here presented pathogenetic mechanisms. In addition, during coronary vessel canalization these drugs may be administered alone or together with a stent-operating procedure to significantly reduce or prevent the pathological outcome of ischemic heart injury.

Acknowledgments

Conflict of Interest Statement

We declare that there are no financial or personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of the manuscript.

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Legends to Figures

Figure 1. Association between extracellular RNA and TNF-α in myocardial ischemia/ reperfusion injury during cardiac surgery. (**A**) Extracellular RNA and (**C**) TNF-α were quantified in plasma from cardiac patients undergoing surgery that was withdraw from radial artery (RA) or coronary sinus (CS) at the indicated time points: T_0 (anesthesia induction – basal level), T_1 (thoracotomy), T_2 (2 min before aortic clamping), T_3 (2 min before aortic unclamping), T_4 (15 min after aortic unclamping), T_5 (30 min after aortic unclamping). Data represent mean ± SEM (n=15; *p<0.05, **P<0.01, ***P<0.001, ns=non-significant). (**B**) Representative analysis of patient´s extracellular RNA, isolated from plasma and subjected to capillary gel electrophoresis, reveals high RNA stability, indicated by the 28S and 18S rRNA bands at each time point.

Figure 2. Release of extracellular RNA during myocardial infarction following I/R. (**A**) Following induction of the *in vivo* cardiac I/R mice model with 60 min ischemia and 180 min reperfusion, plasma extracellular RNA was quantified at the indicated time points. Data represent mean ± SEM (n=6 mice per group; **P<0.01, ***P<0.001). (**B**) After coronary ligation for 60 min without reperfusion, the mRNAs of the following genes were quantified by real-time PCR to deduce their cellular origin: Sarco-/endoplasmic reticulum Ca^{2+}-ATPase (*Serca* – cardiomyocytes), α-smooth muscle actin (*Sma*, smooth muscle cells, SMC), bHLH transcription factor (*Tcf21* – cardiac fibroblast), PECAM-1 (*Cd31* - endothelial cells), colony stimulating factor 1-receptor (*Cd115* – macrophages), neutrophil elastase (*Elane* – neutrophils) and mast cell protease-6 (*Mcpt6* – mast cells). Values are expressed as mean ±SEM (n=6); *P<0.05, **P<0.01, ***P<0.001 *vs GAPDH* expression. The insert indicates the electrophoretic mobility of 28S and 18S rRNA in a representative sample. (**C**) Isolated rat hearts (Langendorff-model) were submitted to I/R (45 min ischemia, followed by 120 min

reperfusion), and extracellular RNA was quantified in the effluent fractions for each time point. Values are normalized to the effluent protein concentration and represent mean ± SD (n=9 hearts) as compared to the minimal values at 3-5 min; *P<0.05, **P<0.01, ***P<0.001. The insert indicates the microparticle-associated total RNA that was quantified from collected perfusate over 120 min and normalized to the effluent protein concentration. Values represent mean ± SD (n=9 hearts); **P<0.01. (**D**) Extracellular RNA was quantified under normoxia (3h) or hypoxia (1h) in supernatants of untreated (-) or TNF-α-treated cardiomyocytes. Values represent mean ± SD (n=6); *P<0.05, **P<0.01.

Figure 3. Extracellular RNA potentiates the release of TNF-α under hypoxia. (**A**) After induction of the *in vivo* cardiac ischemia/reperfusion (I/R) mice model, sham-operated or coronary occluded animals with the following time intervals of I/R (in min): 60/0, 60/2 and 60/120, respectively, were left untreated (Buffer) or were treated with RNase1 (100 μg/mice) or RNase-inhibitor (RI, 80 U/mice) as indicated, and plasma TNF-α was quantified at the end of the respective experiment. Data represent mean ± SD (n=6-8 mice per group); *P<0.05, **P<0.01, ns=non-significant. (**B**) Following induction of the *in vivo* cardiac I/R (60 min/120 min) mice model, the presence of TNF-α in cryosections from heart tissue was demonstrated by confocal microscopy. Representative merged images from immunofluorescence staining for TNF-α (red), F-actin (green) and nuclear DNA (4′,6-diamidino-2-phenylindole [DAPI], blue) are displayed. All images were obtained under identical conditions of confocal laser beam intensity and exposure time (n=6). (**C**) TNF-α-release was quantified in fractions throughout the first 15 min reperfusion period in the Langendorff heart, submitted to the I/R protocol in the absence (squares) or presence of 10 μg/ml RNase1 (circles) or 2 U/ml RI (triangles). TNF-α values are corrected for the respective flow rate and normalized to the effluent protein concentration and represent mean ± SD (n=3-6 hearts); [#]P<0.001 (I/R *vs.* pre-

ischemic values); *P<0.001 (I/R+RNase1 *vs.* I/R). (**D**) Cardiomyocytes were treated under normoxia (3h) or hypoxia (1h) with RNA, hydrolyzed RNA or RNase1 (10 μg/ml), followed by analysis of TNF-α in corresponding cell supernatants. Values represent mean ± SD (n=3); *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Prevention of ROS production by RNase1. (**A**) After induction of the *in vivo* cardiac I/R mice model, sham-operated or coronary occluded animals with the following time intervals of I/R (in min): 60/0, 60/2 and 60/120, respectively, were left untreated (Buffer) or were treated with RNase1 (100 μg/mice) or RNase-inhibitor (RI, 80 U/mice) as indicated. (**B**) Isolated Langendorff-perfused hearts were either submitted to 3h perfusion under normoxia (control) or exposed to the I/R protocol with the following time intervals of I/R (in min): 45/0, 45/2, 45/120, in the absence (Buffer) or in the presence of RNase1 (10 μg/ml) or RI (2 U/ml) as indicated. The distribution of ROS (dihydroethidium, red) production together with detection of F-actin (green) and nuclear DNA (DAPI, blue) was quantified by immunofluorescent staining in microslices of cardiac tissue in each case. All samples received the same manipulation and all images were obtained under identical conditions of laser beam intensity and exposure time using confocal microscopy. Values represent mean ± SD (n=6-8 per group); *P<0.05, **P<0.01, ***P<0.001. Representative images of multiple experiments are shown.

Figure 5. Extracellular RNA-induced cell death during hypoxia: Promotion via TNF-α and prevention by RNase1 treatment. (**A**) Following the induction of the *in vivo* acute cardiac I/R mice model, infarct size in mice hearts was quantified after coronary occlusion followed by 2h reperfusion in the absence (Buffer) or presence of RNase1 (100 μg/mice) or RNase-inhibitor (RI, 80 U/mice), respectively. Representative pictures show myocardial infarct size in heart sections indicated by dual staining with 2,3,5-triphenyltetrazolium (TTC)

and Evans blue. (**B**) Following the induction of the *in vivo* long-term cardiac I/R mice model, histo-morphological analysis was used to evaluate infarct size in mice hearts in the absence (without I/R) or presence (I/R) of coronary occlusion followed by 14 days of reperfusion. In comparison to sham-operated animals, the other groups were continuously treated with buffer only or with RNase1 (100 µg/mice) or RNase-inhibitor (RI, 80 U/mice), respectively, by infusion via subcutaneously implanted osmotic pump. Representative pictures show myocardial infarct size in heart sections indicated by Gomori 1-step stain. Data represent mean ± SEM (n=6-8 mice per group; *P<0.05, ***P<0.001, ns=non-significant). (**C**) Isolated rat hearts (Langendorff-model) were submitted to perfusion only under normoxia (Control) or submitted to I/R (45 min/120 min) in the absence (Buffer) or presence of 10 µg/ml RNase1 or 2 U/ml RI, respectively. Release of lactate dehydrogenase (LDH) during 120 min reperfusion was measured in the absence (Buffer) or presence of different doses of RNase1 or RI (2 U/ml) as indicated. LDH activity was quantified for each time point and normalized to the tissue weight. Values represent mean ± SEM (n=6-9 hearts); *P<0.05, **P<0.01, ***P<0.001. (**D**) Infarct size was quantified by the TTC reaction in heart sections and expressed as the percentage of necrotic tissue with respect to total ventricular mass in the different treatment groups indicated. Values represent mean ± SD (n=6-9 hearts); **P<0.01, ***P<0.001, ns=non-significant. (**E**) Release of LDH from cardiomyocytes was quantified as an index of sarcolemmal rupture after treatment with RNA (10 µg/ml), hydrolyzed RNA (10 µg/ml), with RNase1 (10 µg/ml), RNase-Inhibitor (RI, 40 mU/ml) or DNA (10 µg/ml), respectively, under normoxia (3h) or hypoxia (1h, grey zone) as indicated. Values represent mean ± SD (n=4); **P<0.01, ***P<0.001, ns=non-significant. (**F**) LDH release was measured in the supernatants of cardiomyocytes after treatment with RNA (10 µg/ml) or TNF-α (20 ng/ml) in the absence (-) or presence of TAPI (10 µM) under normoxia (3h) or hypoxia (1h, grey zone),

respectively. Values represent mean ± SD (n=3, each in triplicate); *P<0.05, **P<0.01, ***P<0.001, ns=non-significant.

Figure 6 Inhibition of metalloproteinases prevents cell death and TNF-α release. (**A**) Release of TNF-α was quantified in the perfusate fractions throughout the 40 min stabilization and first 15 min reperfusion period in Langendorff hearts submitted to I/R protocol alone or after administration (arrow) of TAPI (1 μM). TNF-α values are corrected for respective flow rate and normalized to the effluent protein concentration and represent mean ± SD (n=3 hearts); #P<0.001 (I/R *vs.* pre-ischemic values); *P<0.001 (I/R+RNase1 *vs.* I/R). (**B**) Cardiomyocytes were treated for 3h under normoxia or in the absence (-) or presence of RNA (10 μg/ml, grey zones) with GM6001 (10 μg/ml), TAPI (10 μM), Bay11 (100 μM) or anti-TNF-α (25 μg/ml), respectively, followed by quantification of TNF-α protein in the corresponding cell supernatants. Values represent mean ± SD (n=6); *P<0.05, ***P<0.001. (**C**) LDH release was quantified in the perfusate fractions throughout the 40 min stabilization and first 15 min reperfusion period in Langendorff hearts submitted to I/R protocol alone or after administration (arrow) of TAPI (1 μM). LDH values are normalized to the tissue weight and represent mean ± SD (n=3 hearts); *P<0.05, **P<0.01 vs I/R. (**D**) Cell viability was measured in freshly isolated cardiomyocytes from wildtype (WT) and TNF-α$^{-/-}$ mice under normoxia or subjected to hypoxia (grey zone) for 2h in the absence (-) or presence of extracellular RNA. Data represent mean ± SEM (n=6); **P<0.01, ***P<0.001, ns=non-significant. (**E**) Changes in the cellular mRNA levels of inducible NO-synthase (iNOS) and monocyte chemotactic protein-1 (MCP1) were quantified by RT-PCR in isolated rat hearts perfused for 3h under normoxic conditions (control) or submitted to the I/R protocol alone or in the presence of 10 μg/ml RNase1 or 2 U/ml RI, respectively. (**F**) Changes in cellular mRNA levels of iNOS and MCP1 in cardiomyocytes, untreated (control) or treated with 10

μg/ml extracellular RNA, 10 μg/ml RNase1, 20 ng/ml TNF-α or 10 μM TAPI, respectively, for 3h under normoxia were quantified by RT-PCR. Data in (**E**) and (**F**) indicate changes in the ratio between target and *hprt* mRNA relative to the levels in control cells or left ventricle tissue, respectively, under non-stimulatory conditions and represent mean ± SD (n=6 in cardiomyocytes; n=6 in left ventricle tissue); *P<0.05, **P<0.01, ***P<0.001, ns=non-significant. (**G**) Isolated rat hearts were perfused 3h under normoxic conditions (control) or submitted to the I/R protocol alone or in the presence of RNase1 (10 μg/ml) or RI (2 U/ml), respectively, and protein expression of IκB-α and GAPDH in left ventricle tissue was quantified in western blots. The bar graphs represent IκB-α levels normalized to GAPDH in the different treatment groups. Data represent mean ± SD (n=3); **P<0.01, ***P<0.001, ns=non-significant.

Figure 7 Mechanism of extracellular RNA-TNF-α interplay to induce cardiomyocyte damage and intervention with RNase1 or TACE-inhibitor. Upon hypoxia or cardiac ischemia (**1**), cardiomyocyte damage is accompanied by the release of extracellular RNA (eRNA) (**2**). In turn, eRNA promotes TNF-α-converting enzyme (TACE)-mediated proteolytic shedding of TNF-α from its membrane-associated proform (**3**). Active TNF-α induces intracellular signaling possibly via its receptor (TNF-R1) (**4**), involving NFκB-activation/mobilization and resulting in additional cytokine production, in the downregulation of antioxidant enzymes such as peroxiredoxin-3 (Prdx3) or superoxide-dismutases (SOD) 1-3 and the production of reactive oxygen species (ROS) (**5**). As a consequence, marked mitochondrial damage, loss of ATP-production and pathological oscillations of calcium ions (due to the opening of the mitochondrial permeability transition pore, mPTP) will lead to cell death (**6**). To complete the vicious circle, TNF-α may promote further release of eRNA, which may enhance the ongoing cell destruction (**7**). The administration of RNase1 or TACE-

inhibitor TAPI will significantly limit or prevent the indicated adverse effects and serve as potent interventional strategies in cardio-protection (**8**).
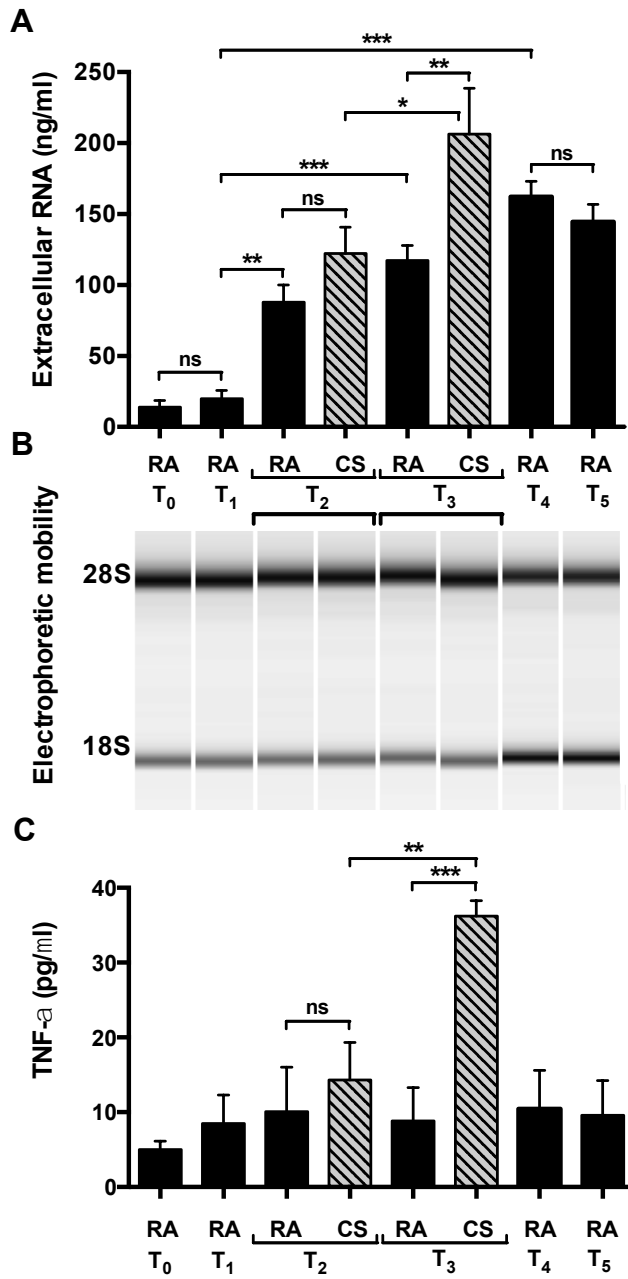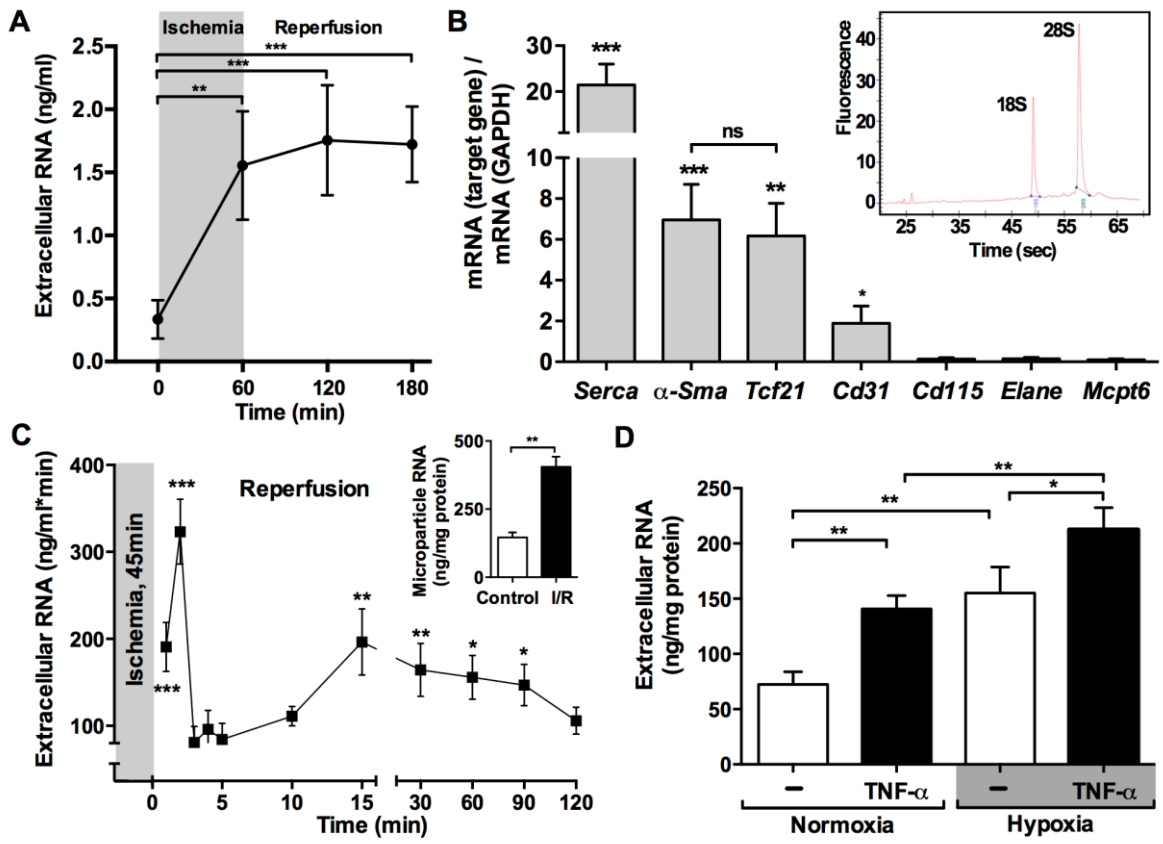
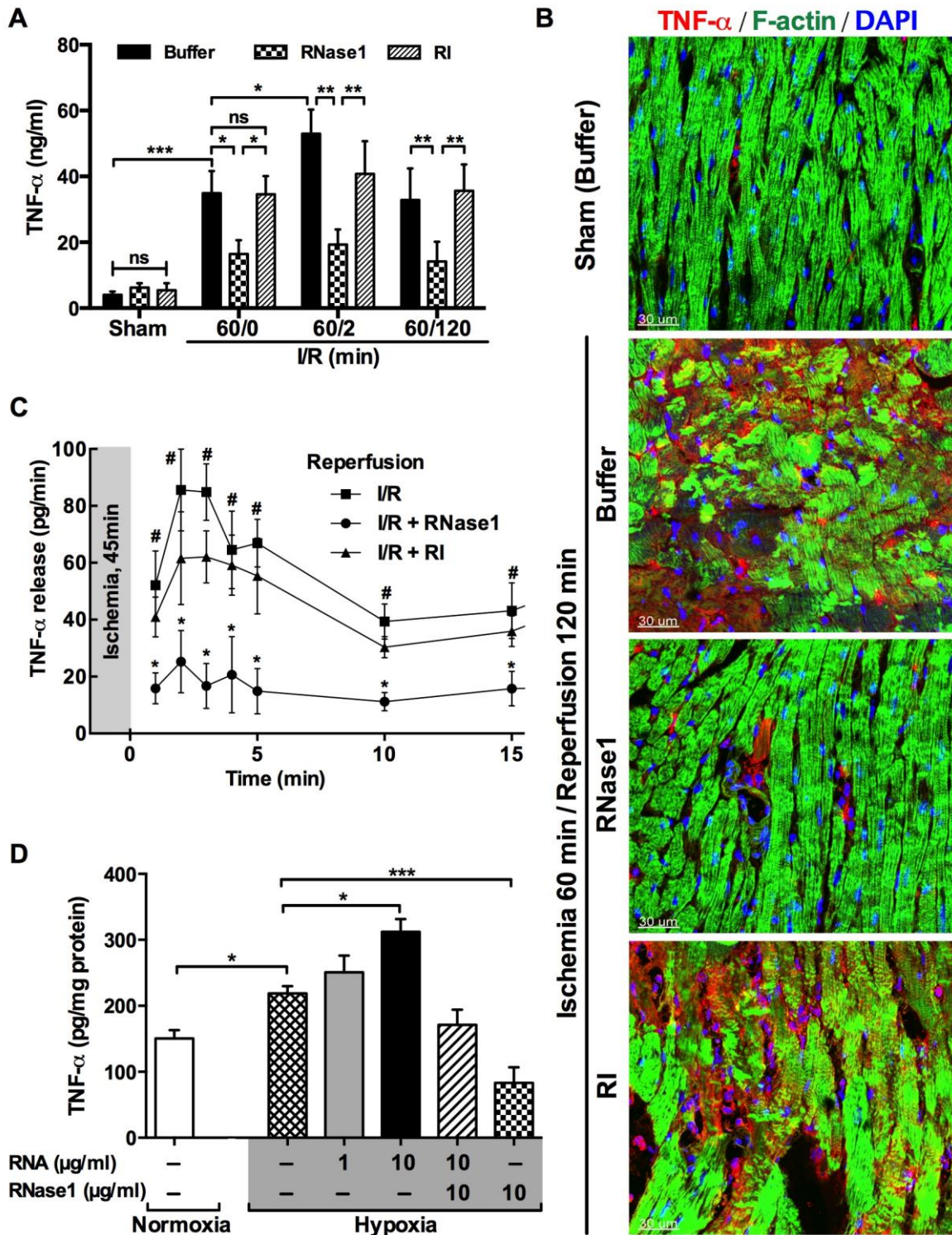Figure 1   Cabrera-Fuentes et al. 2014

Figure 2   Cabrera-Fuentes et al. 2014

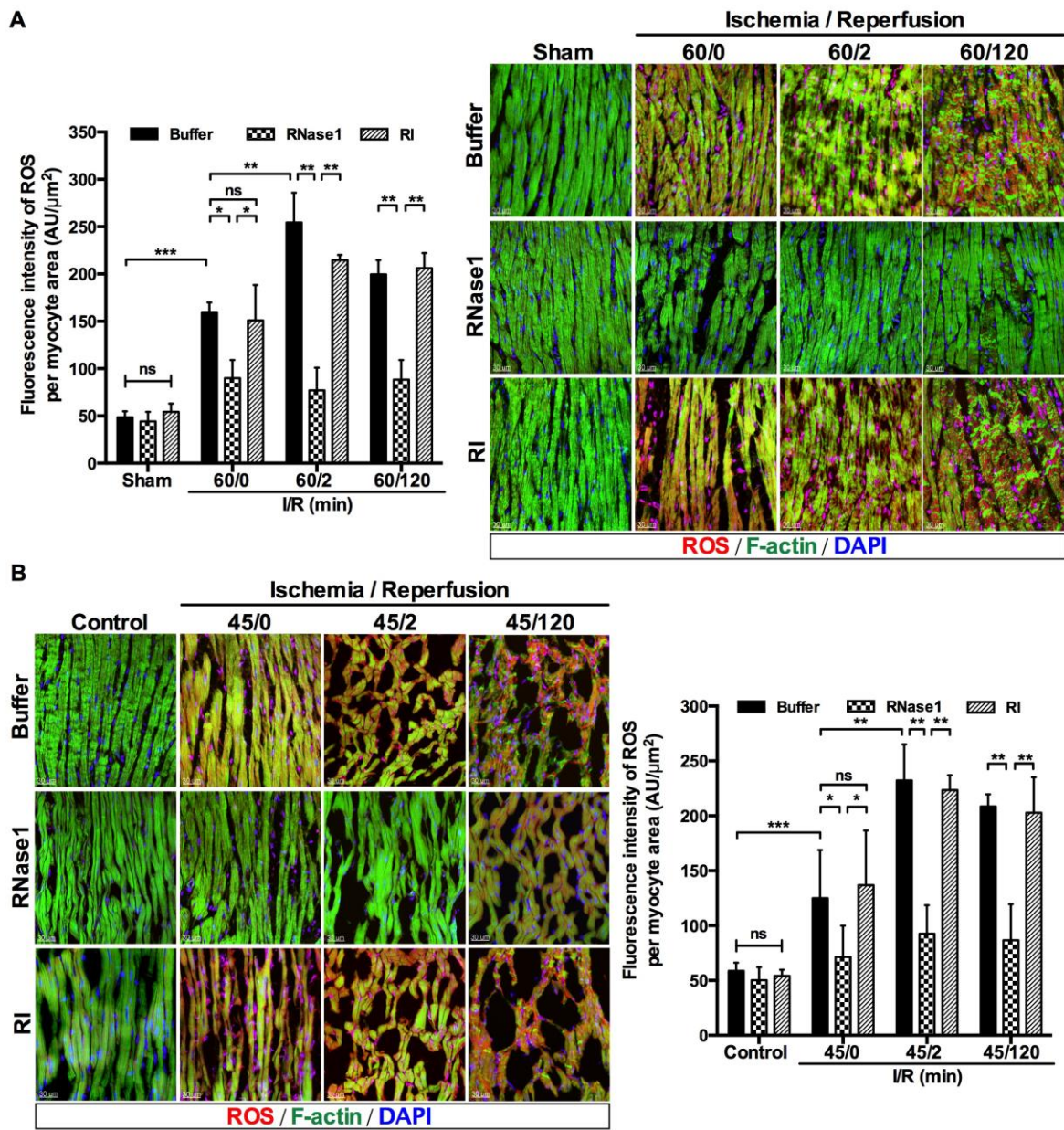Figure 3   Cabrera-Fuentes et al. 2014

Figure 4   Cabrera-Fuentes et al. 2014

ROS / F-actin / DAPI

Figure 5   Cabrera-Fuentes et al. 2014

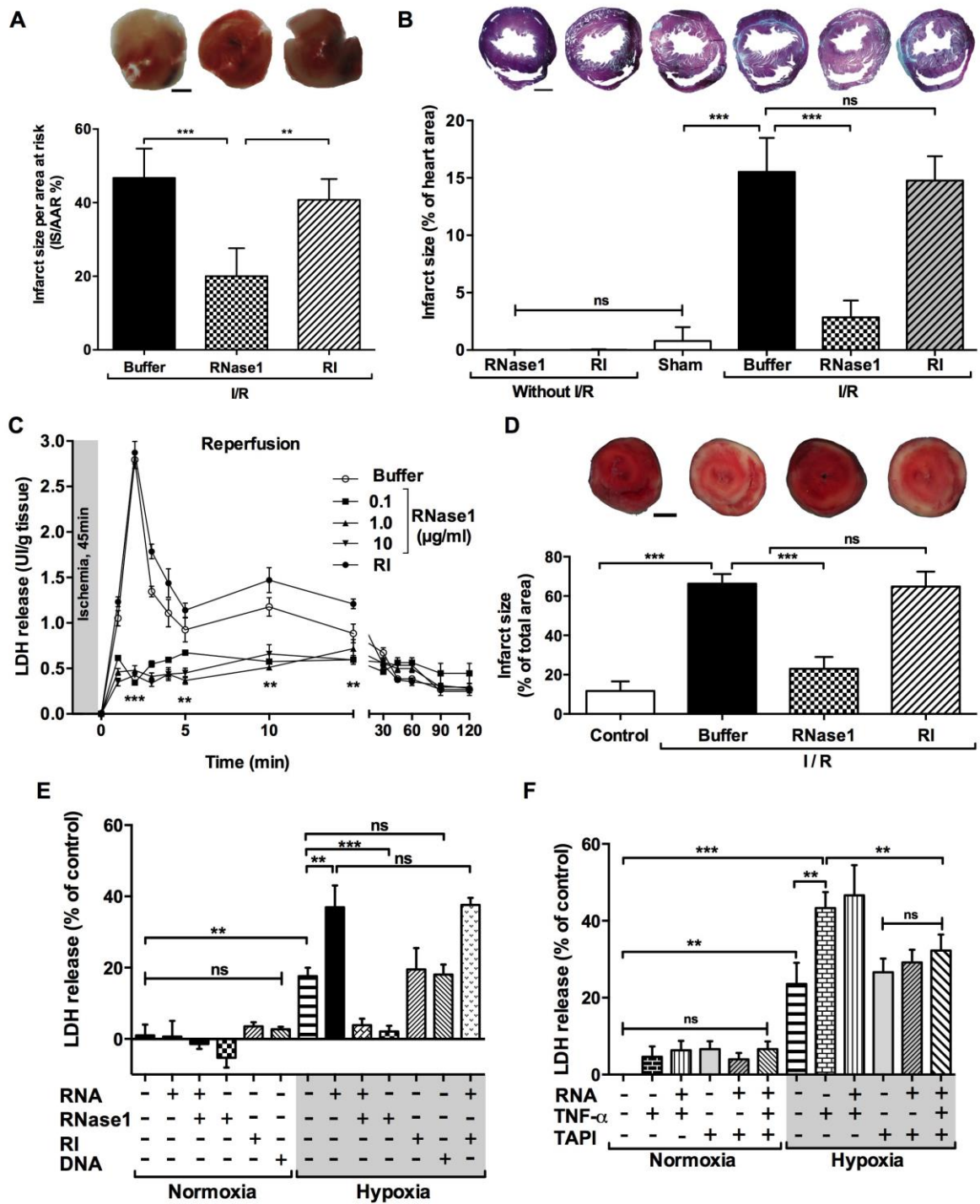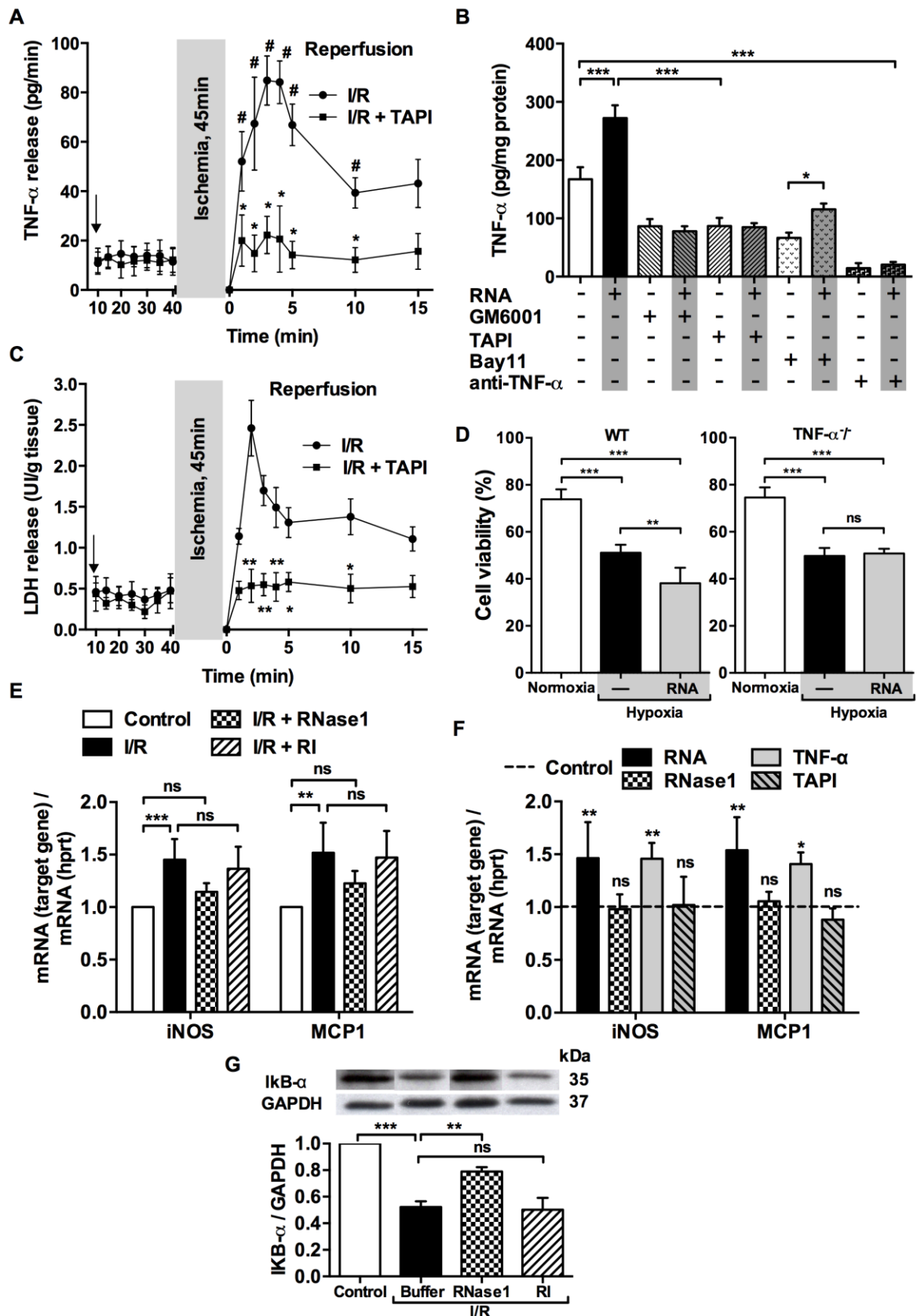Figure 6   Cabrera-Fuentes et al. 2014

Figure 7   Cabrera-Fuentes et al. 2014