Title: Protection from cardiac ischemia-reperfusion injury by epigenetic regulation of NADPH oxidase

Commentary on: "Megakaryocytic leukemia 1 (MKL1) bridges epigenetic activation of NADPH oxidase in macrophages to cardiac ischemia-reperfusion injury" by Yu L et al.

Sean M Davidson[1]* PhD, Derek M Yellon[1] PhD, DSc, FRCP

Running title: MKL1 and cardiac ischemia-reperfusion injury

Affiliation:

[1]The Hatter Cardiovascular Institute

University College london

67 Chenies Mews

WC1E 6HX, London

United Kingdom

***Corresponding author**

Sean M Davidson

Email: s.davidson@ucl.ac.uk

phone: +44 (0) 2034479894

fax: +44 (0)2034479555

Word count: 1,423 in main text excluding title page and references

References: 15

Figures: 1

Ischemia and reperfusion (IR) injury is a complex and multifactorial process that contributes to overall final infarct size after ST-elevation myocardial infarction (STEMI)[1]. Although rapid reperfusion and clinical treatment have led to significant improvements in survival after STEMI, this approach has paradoxically contributed to an increase in patients with post-MI heart failure[1]. As the likelihood of developing heart failure is related to overall infarct size, it is important to identify novel treatments that can target IR injury[2].

The past 30 years have witnessed numerous insights into the multifarious processes of IR injury, and yet, with each step forward, the "Holy Grail" of a clinically effective cardioprotective agent seems to recede further into the distance. For instance, it has been known since well before the millennium that a burst of reactive oxygen species (ROS) production occurs with the re-introduction of oxygen-rich blood to the ischemic myocardium upon re-canalization[3]. However, antioxidants have proven ineffective at limiting infarction. A further step forward was taken with the realization that not all ROS are necessarily damaging. However, mitochondria produce excess ROS during reperfusion, leading to opening of the mitochondrial permeability transition pore (mPTP) and mitochondrial damage[4]. Although mitochondria remain a viable target, attempts so far to target this mechanism have not been successful clinically.

The inflammatory response makes an important contribution to all stages of myocardial IR injury and is another major source of oxidative stress in the reperfused myocardium. Although neutrophils are the "first responders" to injury, they are swiftly followed by the "undertakers" – macrophages, whose task is to remove apoptotic cells and debris[5]. In response to myocardial infarction, large numbers of monocytes emerge from the bone marrow and spleen and invade the damaged myocardium. The first, pro-inflammatory wave of M1-like macrophages peaks at days 3-4, whereas the second wave arrives after about 1 week, differentiates into M2-like macrophages and initiates repair and remodeling. However, it is important to realize that there are already numerous resident macrophages within the healthy heart, accounting for ≈10% of noncardiomyocytes[6].

One of the major sources of ROS in cardiovascular disease are the NADPH oxidases of the NOX family. These include NOX1-3, which produce superoxide when activated, and NOX4, which is unique in that it is constitutively active and produces H_2O_2 rather than superoxide. Mice lacking any of NOX1, 2 or 4 are protected from myocardial IR injury[7]. One of the major sources for NOX-derived ROS is neutrophils, but other inflammatory cell types including macrophages and also cardiomyocytes contribute, as illustrated by the fact that isolated, perfused hearts lacking NOX1 or NOX2 are also protected against IR injury[7]. Circulating macrophages are unlikely to contribute to early ROS generation in the heart, because they do not infiltrate infarcted tissue before 8-12h. However, cardiac-resident macrophages, being already present, may contribute to ROS generation in myocardial IR injury.

The protein MKL1 (megakaryoblastic leukemia 1, also known as myocardin-related transcription factor A or MRTF-A) couples cellular mechanical stress to nuclear transcription. Monomeric G-actin in the cytosol binds MKL1, but when this polymerizes into F-actin filaments, MKL1 is released to translocate to the nucleus where it co-operates with the transcription factor serum response factor (SRF) to modulate transcription[8](**Figure**). Despite its being widely expressed and abundant in the heart, whole-body deletion of *Mkl1* has surprisingly little effect on normal mouse physiology, chiefly impairing mammary gland development and lactation[8]. However, physical stress, such as that triggered by cardiac pressure overload, activates an MKL1-dependent transcriptional response in cardiomyocytes, which protects them against dilated cardiomyopathy[9]. After MI, MKL1 contributes to cardiac remodeling, regulating myofibroblast activation and fibrosis[10]. However, the role of MKL1 in the early phase of IR injury was not previously established.

This issue of the journal contains a detailed examination of the role of MKL1 in the myocardial response to IR injury[11]. Mice lacking MKL1 were protected against IR injury, manifesting smaller infarcts and better cardiac function measured by echocardiography after 45 minutes of coronary artery occlusion followed by 24 hours reperfusion[11]. Interestingly, mice with macrophage- or

cardiomyocyte-specific deletion of *Mkl1* revealed that it is not the MKL1 in cardiomyocytes, but rather the MKL1 in macrophages that is responsible for the increase in ROS that leads to IR injury in this experimental model[11]. A wide variety of *in vitro* and *in vivo* experiments were used to determine that MKL1 in cardiac macrophages induces the transcription of NOX isoforms 1,2 and 4 (but not NOX3). Using chromatin immunoprecipitation, hypoxia followed by reoxygenation was shown to increase MKL1 binding to the promoters of these *Nox* genes in macrophages[11]. MKL1 then recruits the H4K16 acetyltransferase MOF ("Male absent on the first"), which promotes accumulation of active histone acetylation marks, including pan-acetyl H3 (H3Ac), pan-acetyl H4 (H4Ac), and trimethylated H3K4 (H4K4Me3)[11]. Thus, MKL1 appears to activate transcription of *Nox1,2 and 4* genes in macrophages by opening the chromatin structures of the promoters.

MOF was originally identified in fruit flies, where it was found to be essential to ensure appropriate expression of genes on the X chromosome, which are present in only one copy. However, in higher animals it has multiple critical roles in regulating certain genes, including some of those involved in cellular stress. MOF expression is down-regulated in failing human hearts and hypertrophic murine hearts, and transgenic mice overexpressing MOF exhibit less oxidative stress and are protected from cardiac hypertrophy[12].

In addition to using cell-specific transgenic mice, the findings were corroborated using several different pharmacological inhibitors. These included CCG-1423, an MKL1 inhibitor; and MG149, an inhibitor of MOF[11]. Somewhat unexpectedly, daily administration for 3 days prior to infarction conferred only limited protection against infarct formation and provided no benefit in terms of contractile function[11]. This could be explained by Western blot analyses showing that nuclear accumulation of MKL1 and ROS production was only partially suppressed after 3 days. When pre-treatment was extended to 14 days, MKL1 activation and ROS production were completely inhibited, and this resulted in more complete protection against IR injury[11]. Given that MKL1 appears to act via NADPH oxidase, one could ask "why not directly inhibit the NADPH oxidase?". Indeed, this

experiment was also performed using the NOX1/NOX4-specific inhibitor GKT137831 via intragastric lavage for 2 weeks prior to the IR procedure; similar beneficial effects were observed[11].

Despite these encouraging results, it remains unclear whether the requirement for extended treatment was due to the doses of drugs being insufficient, or is indicative of secondary changes that must occur in order to induce cardioprotection. It is also difficult to be certain that the benefit seen with the drugs was due solely to effects on macrophages and not on other cell types such as cardiomyocytes. In addition to fibroblasts, mentioned above, MKL1 plays important roles in neutrophils and the endothelium[13, 14]. The authors do provide evidence that the drugs inhibited MOF in macrophages and not in cardiomyocytes, but it is not entirely clear why this should be the case. It would be informative to investigate whether mice with macrophage-restricted deletion of MKL1 demonstrate additional protection upon administration of the drugs.

In any case, in combination with previous publications, the data suggest that the MKL1/MOF pathway is a promising target for clinical treatment of MI and cardiac remodeling. Notably, no adverse effects were observed during 2 weeks of administration. If, as it seems, MKL1 is mainly involved in stress-specific responses and not in normal physiology[8, 10, 11], it may represent a tractable target.

Of course, outstanding questions remain. For example, how does IR activate MKL1? One possibility is direct mechanical stretch, but a receptor-Rho-mediated pathway could also be involved. Furthermore, as the authors rightly point out, their studies were limited to 24 hours of reperfusion, and it will be important to test for longer-term benefit of these drugs on cardiac remodeling. Lastly, returning to the topic of our introduction, a vital, but challenging step, will be the clinical translation of these observations to the patient. In this regard, it is increasingly recognized that the traditional "one drug, one target" approach has limited ability to protect against IR injury, which consists of many independent processes[15]. Instead, a multi-target approach[15] may be necessary for optimal clinical translation of cardioprotection. If this is indeed this case, then a drug inhibiting the MKL1-

MOF pathway, which appears to have multiple molecular targets across several cell types in the heart, might just fit the bill.

Sources of Funding

SD acknowledges funding from the National Institute for Health Research Biomedical Research Centre (NIHR-BRC) (BRC233/CM/SD/101320).

Conflict of Interest Disclosures

None.

Affiliations

The Hatter Cardiovascular Institute

University College London

67 Chenies Mews

WC1E 6HX, London

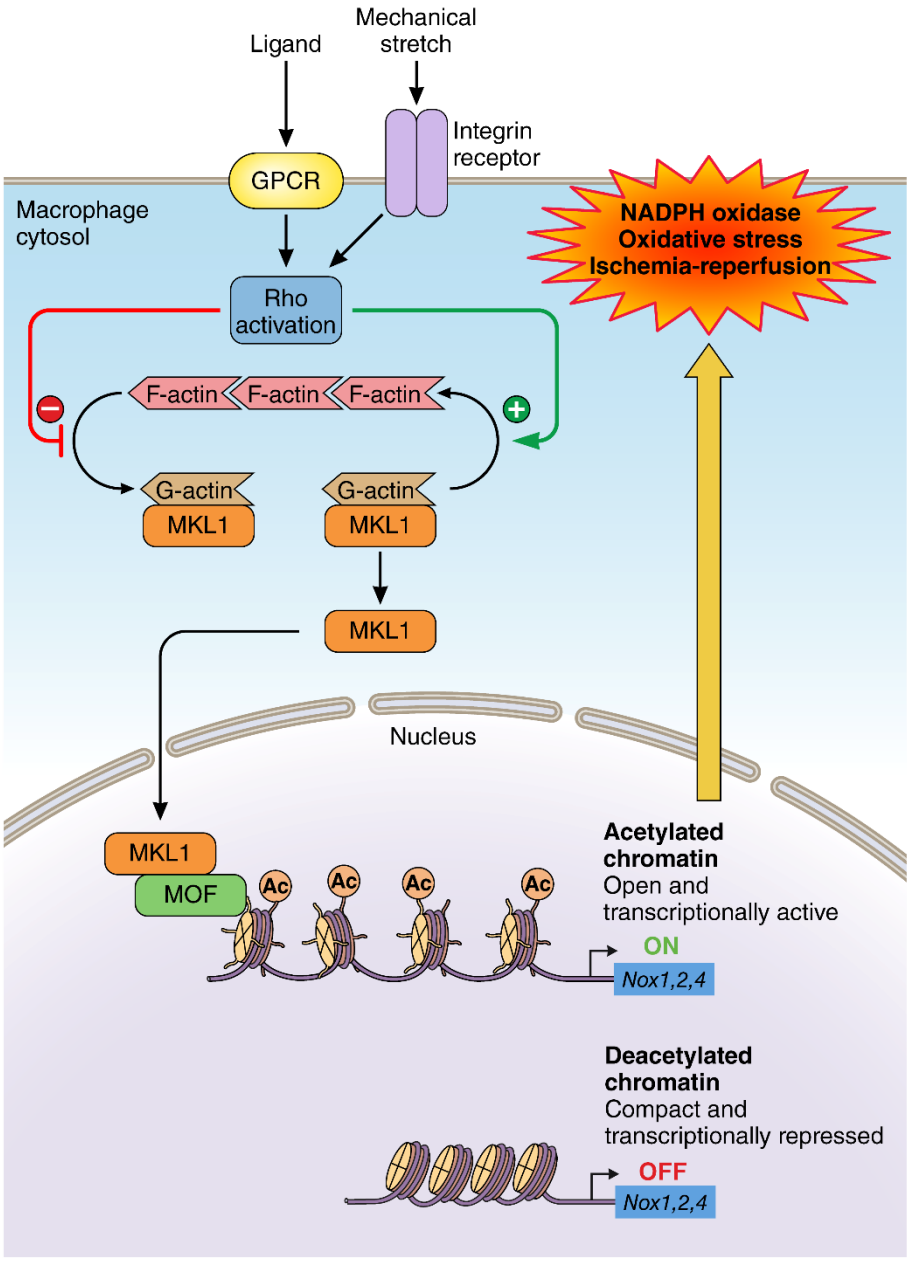
United Kingdom

References

1. Hausenloy DJ and Yellon DM. Ischaemic conditioning and reperfusion injury. *Nat Rev Cardiol*. 2016;13:193-209.
2. Hausenloy DJ, Garcia-Dorado D, Botker HE, Davidson SM, Downey J, Engel FB, Jennings R, Lecour S, Leor J, Madonna R, Ovize M, Perrino C, Prunier F, Schulz R, Sluijter JPG, Van Laake LW,

Vinten-Johansen J, Yellon DM, Ytrehus K, Heusch G and Ferdinandy P. Novel targets and future strategies for acute cardioprotection: Position Paper of the European Society of Cardiology Working Group on Cellular Biology of the Heart. *Cardiovasc Res*. 2017;113:564-585.
3. Granger DN and Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol*. 2015;6:524-551.
4. Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord EN, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa AS, Brookes PS, Davidson SM, Duchen MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T and Murphy MP. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. 2014;515:431-435.
5. Ong SB, Hernandez-Resendiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek XY, Cabrera-Fuentes HA and Hausenloy DJ. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. *Pharmacol Ther*. 2018;186:73-87.
6. Nahrendorf M and Swirski FK. Abandoning M1/M2 for a Network Model of Macrophage Function. *Circ Res*. 2016;119:414-417.
7. Braunersreuther V, Montecucco F, Asrih M, Pelli G, Galan K, Frias M, Burger F, Quindere AL, Montessuit C, Krause KH, Mach F and Jaquet V. Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/reperfusion injury. *J Mol Cell Cardiol*. 2013;64:99-107.
8. Parmacek MS. Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation. *Circ Res*. 2007;100:633-644.
9. Trembley MA, Quijada P, Agullo-Pascual E, Tylock KM, Colpan M, Dirkx RA, Jr., Myers JR, Mickelsen DM, de Mesy Bentley K, Rothenberg E, Moravec CS, Alexis JD, Gregorio CC, Dirksen RT, Delmar M and Small EM. Mechanosensitive Gene Regulation by Myocardin-Related Transcription Factors is Required for Cardiomyocyte Integrity in Load-Induced Ventricular Hypertrophy. *Circulation*. 2018. CIRCULATIONAHA.117.031788
10. Small EM, Thatcher JE, Sutherland LB, Kinoshita H, Gerard RD, Richardson JA, Dimaio JM, Sadek H, Kuwahara K and Olson EN. Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction. *Circ Res*. 2010;107:294-304.
11. Yu LM, Yang G, Zhang XJ, Wang P, Weng XY, Yang YY, Li ZL, Fang MM, Xu Y, Sun AJ and Ge JB. Megakaryocytic leukemia 1 (MKL1) bridges epigenetic activation of NADPH oxidase in macrophages to cardiac ischemia-reperfusion injury. *Circulation*. 2018.
12. Qiao W, Zhang W, Gai Y, Zhao L and Fan J. The histone acetyltransferase MOF overexpression blunts cardiac hypertrophy by targeting ROS in mice. *Biochem Biophys Res Commun*. 2014;448:379-384.
13. Weng X, Yu L, Liang P, Chen D, Cheng X, Yang Y, Li L, Zhang T, Zhou B, Wu X, Xu H, Fang M, Gao Y, Chen Q and Xu Y. Endothelial MRTF-A mediates angiotensin II induced cardiac hypertrophy. *J Mol Cell Cardiol*. 2015;80:23-33.
14. Record J, Malinova D, Zenner HL, Plagnol V, Nowak K, Syed F, Bouma G, Curtis J, Gilmour K, Cale C, Hackett S, Charras G, Moulding D, Nejentsev S, Thrasher AJ and Burns SO. Immunodeficiency and severe susceptibility to bacterial infection associated with a loss-of-function homozygous mutation of MKL1. *Blood*. 2015;126:1527-1535.
15. Hausenloy DJ and Yellon DM. Combination Therapy to Target Reperfusion Injury After ST-Segment-Elevation Myocardial Infarction: A More Effective Approach to Cardioprotection. *Circulation*. 2017;136:904-906.

Figure



Proposed mechanism by which MKL1 (megakaryoblastic leukemia 1, also known as myocardin-related transcription factor A or MRTF-A) in resident cardiac macrophages contributes to ischemia and reperfusion injury. In resting myocardial macrophages, MKL1 is bound to cytosolic G-actin. Mechanical stress and/or G-protein coupled receptor (GPCR)-mediated activation of Rho leads to F-actin polymerization, thereby liberating MKL1 to migrate to the nucleus where it recognizes and binds transcriptional regulatory sites, recruits the H4K16 acetyltransferase MOF ("Male absent on

the first"), and increases the expression of the NADPH oxidases Nox1, Nox2 and Nox3, leading to increased oxidative stress and myocardial damage.