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Should we Target Myostatin, PCSK9 or Their Combination in Ischemia/Reperfusion Injury?

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Introduction

Ischemia/reperfusion (I/R) injury is the consequence of a transient interruption of the blood supply for pathologic, traumatic or surgical reasons, followed by flow restoration with rapid hemodynamic changes in the downstream tissues and organs. This two-step phenomenon is characterized by acute hypoxic moiety, shift from aerobic to anaerobic metabolism, development of acidosis, and increased cell membrane permeability during ischemia; then, tissue reperfusion leads to the activation of prooxidant, inflammatory pathways, and cellular damage affecting lipids, proteins, DNA, mitochondria.

The long-standing assumption is that the oxidative stress damage occurs mainly during reperfusion, when the xanthine oxidase, generated during the hypoxia as byproduct of ATP, produces a burst of superoxide anion and hydrogen peroxide after blood flow restoration and oxygen supply [1].

This general principle appears to be operative in and to complicate both unpredictable (myocardial infarction, stroke) or surgically induced events, as in the case of cardiovascular surgery, visceral organ intervention and organ transplantation. On this basis, many preclinical studies on animal models focused mainly on oxidative damage and encouraged the use of compounds with antioxidant properties as protective strategies [2-6]. However, when translated in the clinical setting, treatments merely based on counteraction of oxidative stress damage gave little results [7] . In line with these observations, it has been reported that while ischemia/reperfusion injury in kidney, liver, heart, and brain of mice was due to succinate accumulation and succinate-driven reactive oxygen species formation, in human kidney transplantation the succinate-driven reactive oxygen formation did not occur; this evidence, together with the absence of allantoin release from the reperfused grafts, suggests that only minimal oxidative stress occurs during reperfusion in transplanted patients [8].

The reason of such discrepancy may be found in species-specific metabolic needs, lifespan, aging processes and their corresponding cell stress response systems. As highlighted by Demetrius [9], in mice – and in general in animal models, which have body mass smaller and life span shorter than humans-the basal metabolic rate per gram of body weight—defined as mass-specific rate—is much higher than in men. Organisms with large mass-specific metabolic rates, as mice, are characterized by high ROS production and weak capacity to maintain homeostasis, whereas humans have a lower mass-specific metabolic rate and a stronger capacity to maintain cellular balance.

In the commented article "Renal Ischemia/Reperfusion Early Induces Myostatin and PCSK9 Expression in Rat Kidneys and HK-2 Cells" [10], authors looked for dysregulation of metabolic mediators in a rat model of surgically-induced renal ischemia/reperfusion injury, as it is often the underlying mechanism of the postsurgical acute kidney injury associated to supra/juxta-renal abdominal aortic aneurysms (AAAs) correction.

Keeping in mind the aforementioned consideration about the different impact of redox fluctuation in rodent and humans, the attention has been focused on two targetable

metabolic mediators, known to be constitutively expressed in the kidney: proprotein convertase subtilisin/kexin type 9 (PCSK9) and myostatin (Mstn).

PCSK9 was discovered in the early 2000s during genetic studies, when mutations linked with PCSK9 gain-of-function were found to associate with familiar hypercholesterolemia; from then on, its loss-of-function resulted to correlate with a better lipid profile and reduced risk of coronary artery disease (CAD). PCSK9 is produced in the liver and, at a lesser extent, in other organs (the kidneys representing an important extrahepatic source). Functionally, once secreted, it binds to the LDL receptor, decreasing its surface expression and thereby increasing circulating LDL levels. As no obvious detrimental effect on health or lifespan in individuals with loss-of-function mutations and very low LDL-C levels has been recorded so far, PCSK9 inhibition successfully started to become a target for CAD, by employing humanized inhibitory monoclonal antibodies [11]. As reviewed by Andreadou and colleagues [12], these agents are now part of the standard armamentarium for CAD risk reduction, while synthetic PCSK9 inhibitor [13] are already used in preclinical studies. Indeed, PCSK9 was found upregulated in myocardial infarction with a negative impact on infarct district and cardiac function [14], and its inhibition in preclinical studies ameliorated infarct size and recovery after myocardial I/R injury [13]. PCSK9 has been shown to regulate other cell receptors, such as the VLDL receptor, apoER2, CD36, and LRP-1. Recently, PCSK9 has been appraised also as a thrombogenic agent: Qi and colleagues [15] demonstrated that PCSK9 promotes platelet activation, presumably through CD36-binding, working as a "DAMP" (danger associated molecular patterns), as referred by Silverstein [16].

Mstn is a TGF-β superfamily member and a known anti-anabolite [17,18]; it has been found upregulated in skeletal muscle cells exposed to oxidative stress conditions [19], after myocardial infarction in hearts of human and mice and has been recognized as a mediator of atherosclerosis progression and vascular inflammation [20]. In kidneys, its enhancement has been observed in the tubulointerstitial compartment during diabetic nephropathy, suggesting a causal role in renal interstitial fibrosis [21].

Since also Mstn loss-of-function didn't display a negative impact on viability and longevity, anti-Mstn treatments have been investigated, particularly in the field of skeletal muscular dystrophy, ageing and cancer-related cachexia. Numerous preclinical studies employing anti Mstn antibodies, Mstn-propeptide with inhibitory function, inhibitor of Activin-receptorIIB and anti-Mstn peptibody gave promising results. However, when translated in humans, they didn't provide the expected outcomes. Reasonably, this may due to different Mstn metabolism in human versus mice and between control subjects and patients' populations. Moreover, a non-selective inhibition of Mstn or of its receptor might cause a disequilibrium in the interaction of several TGF-β superfamily members, perturbating the homeostasis cell program [22].

Some expectations are nowadays fueled by the finding of the more specific Mstn antibody GYM329, created with the "sweeping antibody technology" [23], which provided encouraging results in preclinical studies in monkey, but still to be proven in clinics.

The commented article [10] reported that a 30-minutes supra-coeliac aortic clamping produced I/R injury in rat kidneys; it appeared with higher expression of Mstn and PCSK9 first, within four hours of reflow; then, one day later, signs of focal tubular necrosis, ROS accumulation (expression of N-tyrosine) and Mstn immunopositivity were increased mainly in tubular cells; PCSK9 mRNA was increased and the protein levels reduced in tubular cells but significantly increased in plasma, suggesting a mechanism of transcription/secretion/release of the PCSK9 protein, following, at least in a temporary order, the upregulation of Mstn content.

Hence, the modulation of Mstn and PCSK9 found in kidney during I/R has been verified *in vitro* on HK2 tubular renal cells: Mstn and PCSK9 gene expressions were upregulated early after exposure to ischemia-like condition, to return at the basal level during reperfusion; consistent with the histological findings on rat kidneys, during the whole time-course the increase of Mstn protein content and the depletion of PCSK9 after reperfusion were observed. Prooxidant stress provided by H_2O_2, to mimicry the overproduction of reactive oxygen species due to mitochondria impairment, induced Mstn first and, later on, PCSK9 mRNA; when administered directly to HK2 cell, recombinant Mstn enhanced PCSK9 content that, over time, slightly decreased, suggesting also *in vitro* a mechanism of Mstn-induced production and release. The proof of concept of a Mstn-dependent expression of PCSK9 was provided by reduced levels of PCSK9 in Mstn-silenced cells exposed to ischemia when compared to control cells.

Both Mstn and PCSK9 expressions reflect mitochondrial perturbation: Mstn inhibition through CRISPR/Cas9 gene editing in C2C12 cells mitigated cellular injury by reducing the mitochondrial-dependent apoptotic pathway [24], while PCSK9 both caused and was induced by mtDNA damage in vascular smooth muscle cells [25].

In the commented paper [10], I/R upregulated N-tyrosine, a marker of ROS accumulation, and the nuclear relocation of peroxisome proliferator-activated receptor gamma coactivator-1alpha, a transcriptional regulator of mitochondrial biogenesis and function, indicating the occurrence of mitochondrial imbalance and an attempt to renew the mitochondrial pool, both in kidneys and in HK2 cells.

In HK2 cells both ischemia and reperfusion transiently affected mitochondria, inducing ROS overproduction, mitochondrial membrane potential hyperpolarization and

reduced mitochondrial mass. Cell cycle arrest in G0/G1 phase was observed after ischemia, accounting for an autophagy (mitophagy) process, and recognizing in the early ischemia a priming effect on cell metabolism impairment, previously underestimated in respect to oxidative damage operative during reperfusion.

Integrating the *in vivo* and *ex vivo* observations, the commented paper solidly unveils that 1) renal ischemia is characterized by a combined, local upregulation of Mstn and PCSK9, whose effects are still detectable after reperfusion 2) Mstn overexpression comes first, PCSK9 synthesis and release next and is induced, at least in part, by Mstn.

Yet, the underlying cellular signaling has to be demonstrated, as well as the duration of Mstn and PCSK9 perturbation and their impact on renal function after in I/R injury.

The considerations arisen so far pave the way to ensure mitochondrial protection, when I/R events are predictable, as during surgical I/R, and to cope with I/R injury when it occurs unexpectedly, as in the case of myocardial infarction, ischemic stroke, arterial embolism. Both Mstn and PCSK9, alone, are targetable damage signals; their commitment in a feed forward loop of mitochondrial impairment and inflammation/vascular activation have been already recognized: do they represent a clue to counteract renal I/R injury? Is it possible to act only on the evoked downstream signals or the restraint of Mstn and PCSK9 may constitute also a feedback protection system for mitochondria, seen as "primum movens" of I/R injury? This debate, although still theoretical, will help to define novel strategies to restore the unbalanced metabolic flow and to restrain the fearsome consequence of I/R at large.

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