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EDITORIAL

New insights into cardioprotection, gained by adopting the CAESAR standards of rigor

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Over the past half century, countless therapeutic interventions have been claimed to be cardioprotective in experimental animal models but have failed to limit infarct size in patients with acute myocardial infarction (MI)—one of the greatest translational failures in medical history [6, 7]. The discovery of ischemic preconditioning (IPC) in 1986 [21] ignited great enthusiasm, for it kindled hopes that an effective cardioprotective mechanism had finally been found that was reproducible and would be clinically efficacious. IPC was powerful and consistently limited infarct size in all species and in all animal models tested, and thus was adopted as the gold standard of cardioprotection, the "positive control" for studies purporting to demonstrate the infarct-sparing actions of a therapy. Nothing we knew was more consistent or more effective. Finally, something had been found that promised to "really work". This is why IPC became the major topic of investigation in the field of cardioprotection, driving thousands and thousands of abstract and manuscript submissions, not to mention innumerable grant applications. It had undisputed dominance at cardiology meetings in the 1990s, which routinely included 5–10 sessions devoted solely to preconditioning.

Thirty-six years later, there is no question that IPC has delivered on its promise to be something that "really works" in animal models. The translational problem, however, has always been that, in humans, the onset of MI is unpredictable, making it impossible to harness IPC as a therapeutic intervention, despite its tantalizing power. This sobering fact seemed to be offset by the unexpected discovery that cardioprotection (defined in this essay as infarct size limitation) could also be achieved by applying IPC-like protocols (intermittent coronary occlusions/reperfusions) *after* ischemia, at the time of reperfusion (ischemic post-conditioning) and, even more surprisingly, by performing intermittent occlusions/reperfusions of remote vascular beds, such as the brachial artery, before the onset of myocardial ischemia (in patients undergoing cardiac surgery) or just before reperfusion (achieved by percutaneous coronary interventions in patients with ST-segment elevation MI [STEMI]) (both of these procedures are referred to as "remote conditioning") [15]. These new "twists" on the IPC story again raised hopes that these IPC-related maneuvers would be effective in humans. Unfortunately, more disappointment was on its way, again. Despite the encouraging results of initial smaller studies, subsequent larger, randomized, controlled trials failed to demonstrate the cardioprotective efficacy of post-conditioning and remote conditioning [12, 13, 20]. So, after 50 years of intense basic and clinical research, we still lack an intervention that can be applied to patients with STEMI to achieve limitation of infarct size beyond that afforded by reperfusion alone. Rarely in medicine has so much research yielded so little in terms of clinical therapies.

As discussed recently on the pages of this journal [6], the causes of this colossal failure are multifarious. A major cause is certainly the insufficient rigor of many experimental studies [6, 7]. To overcome this endemic problem, in 2003 we spearheaded the first ever conference focused on rigor (or lack thereof) in studies of cardioprotection, under the auspices of the National Heart, Lung, and Blood Institute [7], and shortly thereafter, in 2010, we developed the CAESAR (Consortium for preclinicAl assESsment of cARdioprotective therapies) consortium to conduct rigorous preclinical studies and promulgate standards of rigor to be adopted in investigations of infarct size limitation [6, 18]. These standards are discussed in more detail in [6] and summarized in Table 1.

Another possible reason for the translational failure is simply that preclinical findings in animal models may not

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Table 1 Standards of rigor implemented and promulgated by the CAESAR consortium [6, 16, 18]

Protocols are standardized and strictly adhered to
Animals are randomized to group assignment
Investigators (both those performing the experimental procedures and those analyzing the data) are blinded
Criteria for inclusion and exclusion of animals are established a priori (i.e., before beginning the study) and not changed during or after the study
The number of animals to be studied is established a priori (i.e., before beginning the study) based on power calculations and is not changed during or after the study
Rigorous statistical analysis is performed by an independent statistician
Exclusions, technical problems, and protocol deviations are carefully reported

be relevant to patients. Do the obvious, enormous differences between animals and humans preclude extrapolation of results from the former to the latter? The answer to this age-old question is still unclear, as no therapy has been unequivocally proven to limit infarct size in humans except reperfusion, but certainly there are plenty of factors that, in principle, can interfere with extrapolation of studies of cardioprotection. Unlike the healthy, adolescent/young animals used in preclinical studies, patients with STEMI have several comorbidities, are middle-aged or old, receive multiple medications, and may have had clinically undetected intermittent ischemia prior to STEMI (which would cause IPC), subtotal coronary occlusion during part of the ischemic phase of STEMI, or intermittent reperfusion (which would cause postconditioning); all of these factors could interfere with infarct size limitation. Among the comorbidities, a highly prevalent one that may obfuscate the protection of IPC is the metabolic syndrome (MS), which is becoming increasingly common in western societies and is associated with glucose intolerance, obesity, hypertension, and dyslipidemia. It should be noted that, at present, the above list of factors that could preclude extrapolation of experimental data on cardioprotection to humans consists of phenotypic or pathophysiological differences; it does not include *genetic* factors.

In this issue of the journal, Kleinbongard et al. [17] report a study that adds a new factor to the aforementioned list, namely, the presence of a genetic background that hinders cardioprotection even in the absence of a phenotype. These authors performed a large study in 62 Ossabaw minipigs, a strain that has a single nucleotide polymorphism encoding for isoleucine (I) rather than valine (V) in the gamma subunit of adenosine monophosphate-activated protein kinase (AMPK) and develops MS when fed a hypercaloric and atherogenic diet [22]. To determine whether this mutation in the *AMPK* gene affected IPC even before any phenotype becomes apparent, the V/V and I/I homozygous genotypes were compared in lean animals fed with normal diet, which did not exhibit any evidence of MS (as documented by body weight and a comprehensive set of blood tests, including glucose and lipid levels). Thus, the only discernible difference between these animals and commonly used pig strains (such as the Göttingen minipigs) was their genotype. The IPC protocol was the same as that shown to be effective in farm pigs (*Sus scrofa*) in the CAESAR consortium [16]. The results showed that neither the V/V nor the I/I genotype exhibited infarct size reduction with IPC, in direct contrast to the powerful IPC protection found in contemporary studies by these authors in Göttingen minipigs. A thorough genomic comparison was then performed between the Ossabaw minipigs and two popular preconditionable strains (Göttingen minipigs and *Sus scrofa*), which revealed differences in numerous genes, including those encoding the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) proteins and mitochondrial proteins, both of which are involved in IPC [15]. The Ossabaw minipigs did not exhibit the increase in STAT 3 phosphorylation during early reperfusion that Heusch's group has previously shown to play a causal role in the IPC protection in Göttingen minipigs [11].

Kleinbongard et al. must be congratulated for a large, well-designed, and well-executed study that no doubt required considerable effort. This work is noteworthy for various reasons. First, it is one of the most rigorous studies of cardioprotection published so far. The authors are to be applauded for adopting the standards of rigor promulgated by the CAESAR consortium [16] (Table 1) and subsequently implemented as a requirement for publication by the editors of *Circulation Research* [3, 4]. Specifically, animals were randomly allocated to treatment; the investigators were blinded; the number of pigs was set a priori on the basis of a careful power analysis performed prior to initiating the study and was not changed during or after the study; protocols were meticulously defined; and a rigorous statistical analysis was conducted. Of note, the authors resisted the temptation to increase the number of pigs a posteriori, after the study was unblinded and some group differences (e.g., between I/I genotype minipigs) were found to almost reach statistical significance—a practice that would have skewed the results. All of these aspects of the study are in accordance with the CAESAR standards [16] and ensure the highest possible level of scientific validity. It is encouraging to observe that the legacy of CAESAR is alive and well [6], and that the emphasis on rigor ignited by that consortium more than a decade ago [7, 18] continues and is actually spreading

ubiquitously, as shown by several recent similar guidelines [8, 9, 14, 19]. This emphasis will greatly reduce contradictory studies, false-positive results, and the attendant confusion that has plagued cardioprotection, which in turn should bring clarity to many apparent controversies, avoid unnecessary clinical trials, and help move the field forward.

An important aspect of the Kleinbongard study is that the authors have clearly separated the effects of the genetic background in the Ossabaw strain from the effects of the phenotype (MS). Since the animals did not exhibit a phenotype of MS (based on body weight and on the analysis of glucose and lipid metabolism), it must be concluded that the failure of IPC to limit infarct size cannot be ascribed to the MS, but instead, is due to as-yet-unknown genetic factors that prevent IPC even before the MS develops. As mentioned above, until now factors invoked to explain the failure to reproduce in humans the findings observed in preclinical studies have usually included age and comorbidities (hypertension, dyslipidemia, diabetes, MS, etc.), i.e., phenotypic factors. Kleinbongard et al. have unveiled a new factor—genetic differences—that can abrogate IPC even in the absence of a specific phenotype.

The obvious question, then, is: what are the genes responsible for abrogating IPC? Answering this question fully will be extremely difficult, and probably impossible. Kleinbongard et al. have made a laudable effort to identify the genetic differences that may account for the failure of Ossabaw pigs to be preconditioned but, as usual in this kind of large-scale, whole-genome studies, they could not pinpoint the gene(s) responsible. Using a comprehensive bioinformatics analysis, they found many genes that differ in Ossabaw pigs vis-a-vis Göttingen minipigs and *Sus scrofa*, but the mere association of these genes with lack of cardioprotection does not enable one to conclude that any or some of them account for the failure to precondition. Associations do not prove causality, although they are useful to plan future mechanistic studies. Establishing cause-and-effect relationships, particularly in pigs, will be a formidable task, one that may not be realistically possible, and so, the precise identity of the gene(s) that interfere with cardioprotection in Ossabaw minipigs will likely remain unknown. What seems clear is that AMPK is not involved in IPC, since neither genotype (I/I and V/V) was amenable to preconditioning.

The findings of Kleinbongard et al. raise several other questions. Is the late phase of IPC also abrogated in Ossabaw minipigs? IPC induces two distinct phases of cardioprotection: an early phase, which occurs within minutes and lasts only a few hours (this is the phase studied by Kleinbongard et al.) and a late, or delayed, phase, which manifests approximately 24 h after ischemia/reperfusion and lasts 42–72 h [1]. The mechanisms for these two phases are different [2]. Early preconditioning is underlain by post-translational modifications of existing proteins, which explains its rapid occurrence and disappearance. By contrast, late preconditioning is caused by activation of a stress-responsive genetic program, which results in the synthesis of several new proteins that confer resistance to ischemia/reperfusion injury [2]. Like early preconditioning, late preconditioning has also been consistently demonstrated in every species tested heretofore, but whether the recruitment of this cardioprotective genetic adaptation is dependent on variations in genotype within the same species is currently unknown. Because of its longer duration, late preconditioning may actually have greater clinical relevance than early preconditioning, and so it would seem important to address this issue.

Another question relates to the various types of preconditioning. Transient ischemia is not the only stimulus that triggers a preconditioning response. A host of chemical agents (e.g., adenosine, bradykinin, nitric oxide, carbon monoxide, etc.), stresses (e.g., heat shock, hypoxia, etc.), cytokines, chemokines, noxious compounds (e.g., endotoxin), and other stimuli can also promote a switch to a cardioprotective phenotype analogous to that triggered by transient ischemia [2]. Although the molecular mechanisms for these non-ischemic forms of preconditioning appear to be similar to those of IPC, it would be interesting to examine whether the genetic factors that abrogate IPC in Ossabaw minipigs also abrogate other types of early PC in these animals.

Perhaps the most surprising result of the Kleinbongard study is that genetic variations associated with pig *strains* are sufficient to block the cardioprotective machinery responsible for IPC, which has been demonstrated in every species tested and is the most reproducible cardioprotective mechanism known [2]. These genetic differences *among* species do not abolish IPC but genetic differences *within* the same species are sufficient to do so and was unexpected. If the same is true for humans, this would imply that the response to IPC may be highly heterogeneous, with some patients exhibiting protection and others failing to do so. And since IPC is the archetypical form of cardioprotection, the response to cardioprotective therapies, in general, may also be highly heterogeneous in humans, which would make clinical trials even more difficult unless non-preconditionable genotypes are identified and excluded.

In summary, the study by Kleinbongard et al. is an important advance in our understanding of IPC. It reveals heretofore unexpected complexities in this cardioprotective mechanism, namely, that it is underlain by genetic factors that are as yet unknown, that cannot be inferred from phenotypic features (since the MS had not yet developed in the Ossabaw minipigs), and that can vary significantly among individuals within the same species. Pinpointing these factors will be extremely difficult, if not impossible. This sober realization adds another obstacle to the already arduous pathway for translating infarct size limitation from animal models to humans. Apart from reperfusion,

unequivocal evidence that infarct size can be reduced in humans by IPC or any other intervention is still lacking. It is also not clear if such evidence will be forthcoming in the near future. Fortunately, over the past three decades, the incidence of STEMI has decreased enormously (by ~ 70%) and its prognosis has improved dramatically, with a current aggregate mortality of ~ 1–2% and an incidence of heart failure of < 7% at 1 year even among patients with left ventricular dysfunction in the acute phase [5, 10], which means that most patients do not require a cardioprotective therapy. Undoubtedly, however, there are, and will always be, subsets of patients (e.g., those with one or more prior MIs, large anterior STEMI, etc.) who do need therapies that limit myocardial damage to improve their prognosis. Clinical trials must target these high-risk populations.

The phenomenon of preconditioning has fascinated the scientific community for almost four decades because it has revealed that the heart has an amazing ability to respond to stress by changing its phenotype very quickly (within minutes) in a manner that protects it from injury. If this powerful natural mechanism could be harnessed for therapeutic purposes, the benefits would be vast. The study by Kleinbongard et al. adds a new dimension to this puzzle. It reminds us that despite the enormous advances in our understanding of preconditioning since its discovery in 1986 [21], much remains to be learned about the molecular and cellular mechanisms that underlie this remarkable adaptation. The work of Kleinbongard and colleagues also epitomizes the high level of rigor and scientific validity that are attained when studies are conducted in accordance with the CAESAR standards (Table 1). It is hoped that the emphasis on rigor spearheaded by CAESAR over a decade ago will continue to permeate the field of cardioprotection, for this is the only way forward.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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