

Edward D. Verrier, M.D.

- Cellular and Molecular Mechanisms of Ischemia-Reperfusion Injury



AWARDS

American College of Surgeons

- National Committee on Trauma Competition, First Place
- Region X Committee on Trauma Competition, Finalist
- Washington State Chapter Henry Harkins Resident Research Award, First Place
- Washington State Chapter Henry Harkins Scientific Presentation Award

American Heart Association

- Vivian Thomas Young Investigators Award in Cardiothoracic Surgery, Finalist

Helen and John Schilling Resident Research Symposium Awards

Thoracic Surgery Foundation For Research and Education Fellowships

Western Thoracic Surgery Association Sampson Resident Research Award

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Thoracic Surgery Foundation

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The vascular endothelium has a principal role in several of the biologic events that affect the preoperative, operative, and postoperative course of nearly all surgical patients. In response to injury, endothelial cells become activated, releasing or expressing a number of inflammatory mediators that enhance leukocyte adhesion, promote coagulation and induce vasoconstriction. These responses to injury are beneficial to the patient when limited to localized areas of infection or tissue disruption. During severe systemic illness (for example, cardiopulmonary bypass, sepsis, or shock), inflammatory reactions may become generalized, however, initiating a distinct pathologic state called the "Systemic Inflammatory Response Syndrome" (SIRS). Systemic inflammatory reactions in general cause damage to tissue, which leads to organ dysfunction.

around reperfused tissue. Because restoration of oxygen delivery to ischemic tissue is critical to survival, a substantial amount of research in the last decade has focused on treating or preventing this important consequence of reperfusion.

In our laboratory, we have examined the molecular mechanisms of *regional* I/R injury that complicates cardiothoracic surgical procedures, and *systemic* I/R injury that is the result of resuscitation in the trauma patient with hemorrhagic shock. The scope of our research includes the study of basic biologic processes at molecular, cellular and physiologic levels, and the examination of the pathophysiologic mechanisms of I/R injury. Our goal is to translate an understanding of the molecular mechanisms of I/R injury into applications for clinical practice.

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Ischemia-reperfusion injury: Ischemia/reperfusion (I/R) injury contributes significantly to morbidity and mortality in surgical patients. I/R injury is the principal pathogenetic event in stroke, complications of peripheral vascular disease, hemorrhagic shock, and early transplant graft dysfunction. Paradoxically, reperfusion of oxygen-deprived tissue, the mainstay of therapy for ischemia, causes further tissue injury by inciting a deleterious inflammatory reaction in and

The cellular and molecular mechanisms of endothelial cell activation during I/R injury are complex. These mechanisms result in tissue factor expression (leading to microvascular thrombosis and disseminated intravascular coagulation [DIC]); neutrophil adhesion secondary to upregulation of neutrophil adhesion molecules on activated endothelium (for example, E-selectin); and leukocyte activation and chemotaxis caused by the release from I/R injured endothelium of

chemokines (for example, Interleukin-8) and growth factors. One component of our research is based on the transcription factor, NF- κ B, that regulates transcription and expression of the genes that encode these proteins.

NF- κ B is composed of subunits from the NF- κ B/Rel family of proteins. Five distinct DNA-binding proteins of the family, p50, p52, p65 (also known as RelA), c-Rel, and RelB, are involved in mammalian transcription. Members of this family are defined by the presence of a highly conserved region of approximately 300 amino acids called the "Rel homology domain," which bears the DNA binding site, located in the amino-terminus half of the domain.

During I/R injury, NF- κ B activation may be preceded by signal transduction through a cytoplasmic molecule, p38, of the MAP kinase family of signal transduction proteins. Once activated in response to environmental changes surrounding a cell, p38 in turn activates transcription factors leading to changes in gene expression in cardiac cells, endothelial cells of coronary vessels, or inflammatory cells such as macrophages.

Thrombin is generated during reperfusion and may mediate reperfusion injury. Thrombin interacts with a specific cell receptor, protease-activated receptor-1 (PAR-1), present on endothelial cells, cardiac myocytes, and macrophages, signaling changes in gene expression in these cells. Complement chemotactic fragments, C3a and C5a, are also generated during reperfusion of ischemic tissue and, with thrombin, may be the initiating signals of I/R injury.

The specific aims of our research are: (1) Determine the molecular pathways that lead to NF- κ B activation during ischemia and reperfusion; (2) determine the role of NF- κ B-mediated gene transcription in regional and systemic I/R injuries; and (3) identify novel therapies that block NF- κ B activation only during I/R injury, preserving the capacity of the cell, and the patient within whom it resides, to respond to other injuries (e.g., sepsis).

Experimental techniques: We utilize cultured cells to examine molecular mechanisms that are involved in the response to I/R injury. Although cell culture is a highly artificial system, it allows us to examine in precisely controlled conditions specific questions about the effects of hypoxia and reoxygenation on molecular pathways in human cells.

In addition, cell culture gives us the capability to move DNA sequences into human cells in a controlled fashion to deduce cellular mechanisms of activation based on the effect of the protein encoded by the trans-

fect DNA on cellular function. Finally, by employing differential array and DNA microchip technology, we can identify and characterize novel protein kinases or transcription factors that, in concert with NF- κ B, regulate the cellular response to hypoxia and reoxygenation. Interpretations of findings *in vitro* are provisional, however, until they can be confirmed *in vivo*.

We have developed several animal models of regional and systemic inflammatory responses induced by I/R injury. We have also included in our experimental repertoire *ex vivo* perfusion of hearts by the method of Lagendorf. Recently we have found that rabbit hearts made ischemic by transient coronary artery ligation, express large amounts of tissue factor after release of the ligature and reperfusion of the ischemic segment. Furthermore, we have recently reported that inhibition of IL-8 significantly blocks myocardial I/R injury.

We have developed and utilize a mouse model of myocardial I/R injury. A well-defined I/R injury is induced in mouse hearts by transient occlusion of the left anterior descending coronary artery. Following reperfusion we determine the size of the infarcted region to quantify the magnitude of cardiac I/R injury. Although the mouse myocardial I/R injury model is technically challenging and is performed in only two other laboratories in the U.S., use of transgenic or gene knockout strains allows us to examine the effect of specific genotypic changes (and thus phenotypic changes) on myocardial I/R injury.

For example we have examined mice that have been genetically engineered to lack PAR-1 (PAR-1 knockouts; or PAR-1 $-/-$). Compared to wild-types, PAR-1 knockouts develop a significantly smaller infarct after myocardial I/R injury — confirming, as we have postulated, that thrombin (through its interaction with PAR-1) plays a necessary role in the pathogenesis of I/R injury. Furthermore, based on evidence we have developed with regard to signaling pathways involved in myocardial I/R injury, we have been able to pharmacologically reduce infarct size in our mouse model of I/R injury. Blockade of p38 activity with a proprietary compound significantly attenuates infarct size after ischemia and reperfusion compared to mice treated with vehicle alone. Thus, we have been able to apply what we have determined about the basic science of myocardial I/R injury to potential clinical development.

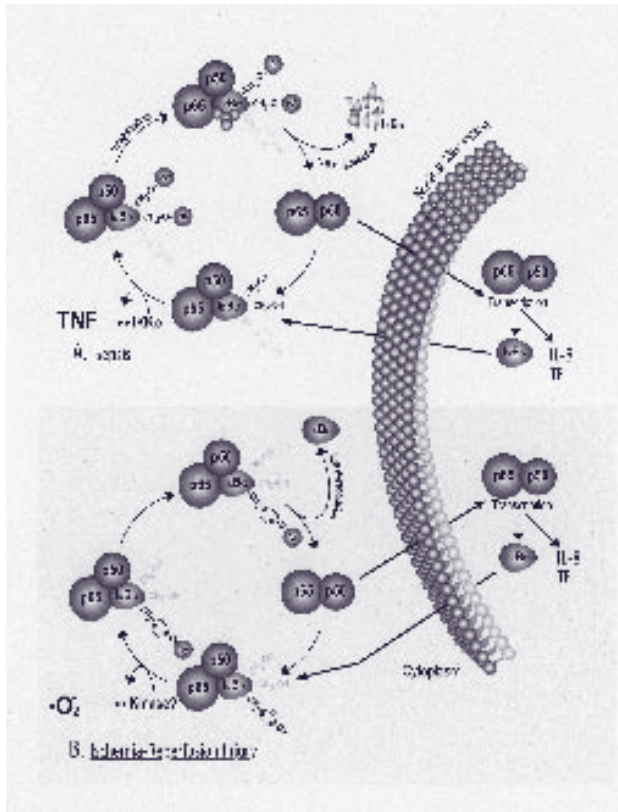


FIGURE 1: Parallel pathways of NF-κB activation. In (A), septic stimuli, such as TNF-α (or, IL-1 or LPS), activate transmembrane signaling pathways in responsive cells leading to the phosphorylation of a serine/threonine kinase, IKKα. This kinase in turn phosphorylates IκBα on serine residues 32 and 36. IκBα is an inhibitor of NF-κB, but upon phosphorylation undergoes degradation. Degradation of Ser-phosphorylated IκBα requires the addition of ubiquitin molecules that target proteins for degradation in proteasomes.

After IκBα degradation, NF-κB, consisting of two subunits, p65 and p50, then translocates to the nucleus, where it binds to specific DNA sequences in the 5'-flanking region of several genes that encode proteins mediating inflammatory reactions (for example, IL-8), coagulation (for example, tissue factor [TF]), and immunologic reactions. Of interest, NF-κB regulates transcription of new IκBα, which functions in a negative feedback loop to down-regulate this particular cellular response.

In (B), reactive oxygen intermediates activate signaling pathways yet to be determined that lead to tyrosine phosphorylation of IκBα. The tyrosine kinase (or kinases) responsible for this reaction has not yet been identified. Tyr-phosphorylated IκBα, in contrast to Ser-phosphorylated IκBα, dissociates from NF-κB without degradation. NF-κB subsequently translocates to the nucleus to promote transcription of a similar set of genes as shown in (A), including IκBα.

This figure shows the molecular basis for the inflammatory reaction induced by ischemia-reperfusion injury, or any other injury in which reactive oxygen intermediates are formed. The figure also indicates that it may be possible to suppress an inflammatory reaction associated with ischemia-reperfusion injury that may be detrimental to the patient, without blocking the patient's ability to generate an inflammatory reaction when required to contain microbial invasion.

RELATED PUBLICATIONS

1. Griscavage, JM; Wilk, S; Ignarro, LJ. Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-κB. *Proc Natl Acad Sci USA*, 1996; 93:3308-3312.
2. Boyle EM, Jr; Kovacich JC; Canty TG, Jr; Morgan EN; Verrier ED; Pohlman TH. Inhibition of NF-κB nuclear localization prevents E-selectin expression and the systemic inflammatory response. *Circulation*, 1998; 98(19 Suppl):II282-8.
3. Boyle EM, Jr; Kovacich JC; Hébert C; Canty TG, Jr.; Morgan EN; Pohlman TH; Verrier ED. Inhibition of interleukin-8 blocks myocardial ischemia-reperfusion injury. *J Thor Cardio Surg*, 1998; 116:114-121.
4. Sato TT; Kovacich JC; Boyle EM, Jr; Haddix TL; Weintraub A; Pohlman TH. CD14-dependent activation of human endothelial cells by bacteroides fragilis outer membrane. *J Surg Res*, 1998; 74(2):103-11.
5. Kovacich JC; Boyle EM, Jr; Morgan EN; Canty TG, Jr; Farr AE; Caps M; Frank N; Pohlman TH; Verrier ED. Inhibition of the transcriptional activator protein nuclear factor κB prevents hemodynamic instability associated with the whole-body inflammatory response syndrome. *J Thor Cardio Surg*, 1999; 118(1):154-62.
6. Canty TG, Jr; Boyle EM, Jr; Farr AL; Morgan EN; Verrier ED; Pohlman TH. Oxidative stress-induced NF-κB nuclear localization without IκBα degradation: An alternative pathway for human endothelial cell activation. *Circulation*, 1999; 100(19 Suppl):II361-4.
7. Morgan EN; Boyle EM, Jr; Yun W; Griscavage-Ennis JM; Farr AL; Canty TG, Jr; Pohlman TH; Verrier ED. An essential role for NF-κB in the cardioadaptive response to ischemia. *Ann Thorac Surg*, 1999; 68:377-382.
8. Morgan EN; Boyle EM, Jr; Yun W; Kovacich BS; Canty TG, Jr; Chi E; Pohlman TH; Verrier ED. Platelet-activating factor acetylhydrolase prevents myocardial ischemia-reperfusion injury. *Circulation*, 1999; 100(19 Suppl):II365-8.
9. Pohlman TH; Harlan JM. Adaptive responses of the endothelium to stress. *J Surg Res*, 2000; 89(1):85-119.
10. Erlich JH; Boyle EM; Labriola J; Kovacich JC; Santucci RA; Fearn C; Morgan EN; Yun W; Luther T; Kojikawa O; Martin TR; Pohlman TH; Verrier ED; Mackman N. Inhibition of the tissue-factor-thrombin pathway limits infarct size after myocardial ischemia-reperfusion injury by reducing inflammation. *Am J Pathol*, 2000; 157(6): 1849-62.

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