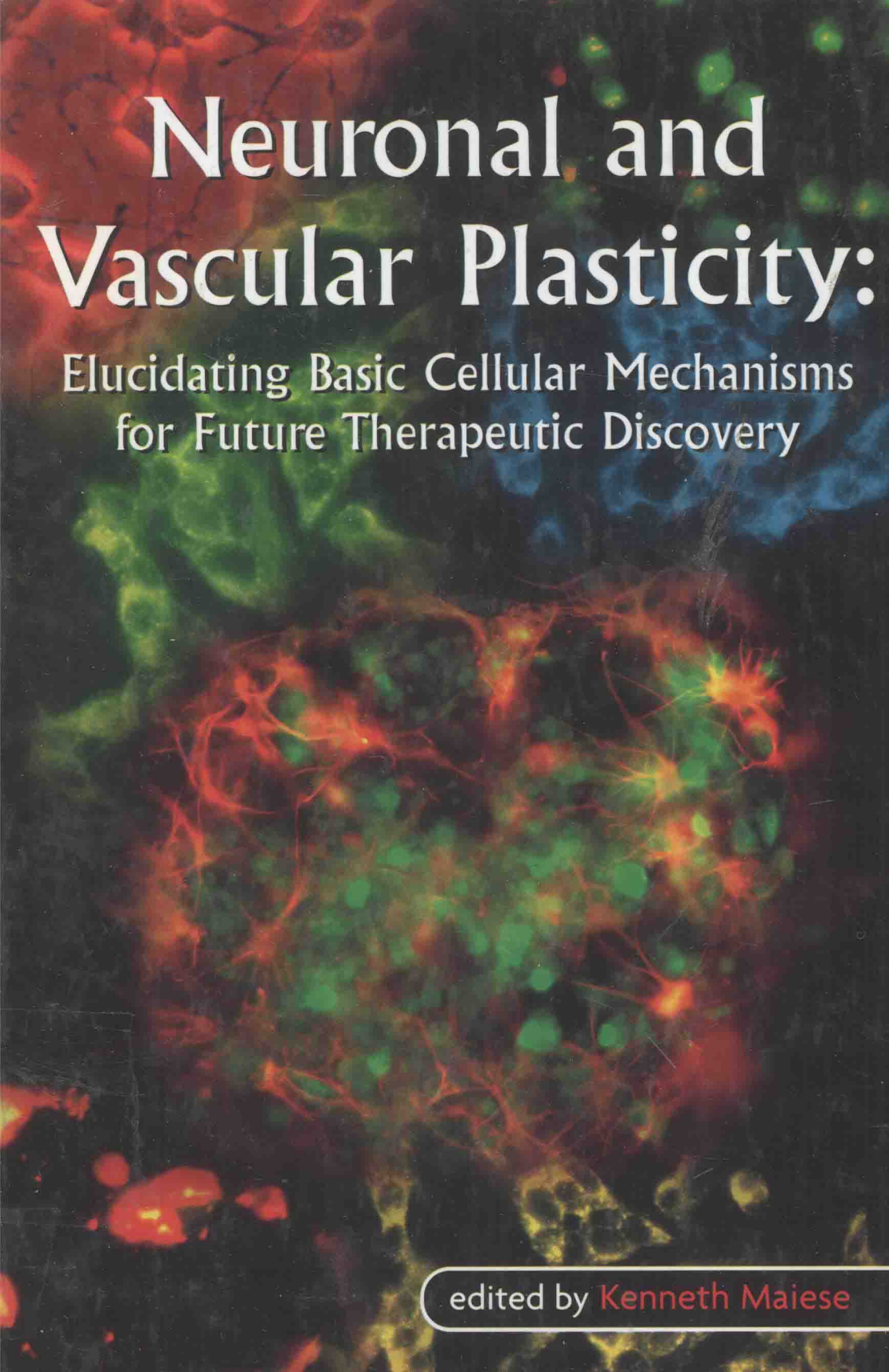
Neuronal and Vascular Plasticity:

Elucidating Basic Cellular Mechanisms for Future Therapeutic Discovery



edited by Kenneth Maiese

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DEDICATION

This work is dedicated to the highly recognized individuals who devoted their time and talents to contribute to this monograph and to our students and colleagues who hopefully will benefit from this work in their endeavors to translate knowledge from basic cellular investigations into viable strategies to treat a wide range of neurodegenerative disorders.

This book also is especially dedicated to my wife, mother, friends, and colleagues for their unending support and patience.

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PREFACE

Galen in the 2nd century AD could be considered one of the earliest researchers who attempted to bridge the gap between basic science and clinical medicine. Galen is given initial credit for the recognition that vital organs of the body are exquisitely dependent upon the intact function of the circulatory system. The doctrines of Galenic physiology stated that blood was produced in the liver, flowed to the heart to obtain "vital spirits", and subsequently bathed the brain to gain "animal spirits".

The "vital spirits" described by Galen were later disclosed to consist of oxygen. Oxygen was discovered independently by Schiele in Sweden and by Priestly in England. It was named oxygen (acid-former) by Antoine Lavoisier (1743—1794) of France. Lavoisier made significant medical discoveries concerning oxygen's role in respiration. In animal experiments, Lavoisier and others discovered that anoxia could rapidly lead to death.

The initial work by these investigators helped provide direction for modern clinical science and the treatment of disease, especially concerning disorders of the nervous system. Remarkably, our understanding of human disease continues to grow at an exponential rate. At times, the accumulation of knowledge of the cellular components of clinical disease exceeds all prior expectations held just a few years ago, such as evidenced by the recent cloning of the human and mouse genomes.

Despite theses advances, both biomedical scientists and clinicians sometimes are at a loss to recognize the crucial link between basic science discovery and the development of therapeutic regiments for clinical disease. In particular, if one focuses upon the central nervous system, greater understanding of the mechanisms of neuronal and vascular survival do not on the surface always appear to further the cause for efficacious drug discovery. For example, agents that eventually make their way through clinical trial investigations more often than not fail to offer safe and effective therapy against a targeted disease. Yet, it is the precise elucidation of the cellular and molecular pathways that determine cellular injury that will offer the greatest potential to either prevent or reverse central nervous system disability. In addition, given the complexity and interplay of the cellular micro-environment, strategies that seek to develop "silver-bullet" agents will most likely continue to disappoint the advocates of such protocols.

The goal of this monograph is to address novel repair mechanisms for cellular injury and integrate current knowledge of basic disease mechanisms of the brain with clinical approaches. Understanding the crucial link between

basic science discovery and the development of therapeutic regimens for clinical disease offers the greatest potential to either prevent or reverse central nervous system disability. "*Neuronal and Vascular Plasticity: Elucidating Basic Cellular Mechanisms for Future Therapeutic Discovery*" is authored by internationally recognized researchers and physician scientists to integrate mechanisms of cellular brain injury and repair with clinical approaches and potential "state of the art" treatment strategies.

An especially attractive aspect of this book is its focused, but comprehensive format that addresses the complexity and potential of the cellular micro-environment for self repair in a manner that is designed to "push the envelope" for new clinical strategies. Chapters cover a broad range of topics, such as the use of embryonic stem cells for restorative cognitive and motor function, investigating the plasticity of cholinergic neurons through microarray analysis, evaluating the molecular mechanisms of ischemic-induced angiogenesis, assessing acute neuronal injury through individual synaptic transmission, modulating the plasticity of the nervous system during acute and chronic toxin exposure, exploiting the potential of reproductive steroids as endogenous neuroprotectants, and furthering the role of the metabotropic glutamate system for both neuronal and vascular cytoprotection.

Offering a concise and relevant approach for translating basic and clinical research into viable therapeutics for both acute and chronic neurodegenerative disease, this monograph is designed to serve as a strong reference for those entering the clinical neurosciences as well as for those established in the neurosciences. In this regard, both clinicians and scientists will hopefully gain further insight into the methods of translating both basic and clinical research into viable therapeutics for degenerative diseases. To achieve such a perspective, we will have come full circle to the initial work of Galen who sought to bridge the gap between basic science and clinical medicine.

Kenneth Maiese

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TRANSFORMATION INTO TREATMENT: NOVEL THERAPEUTICS THAT BEGIN WITHIN THE CELL

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Introduction

Neuronal and vascular injury associated with several disease entities, such as Alzheimer's disease, Parkinson's disease, and cerebrovascular disease was initially believed to be irreversible. Yet, it has become increasingly evident that either acute or chronic modulation of the cellular and molecular environment within the brain can prevent or even reverse cellular injury. Irrespective of the initial insult to the nervous system, the activity and interplay among specific cellular signal transduction pathways in a cell will ultimately determine the extent of injury to the brain. In order to develop rational, efficacious, and safe therapy against neurodegenerative disorders, one must first elucidate potentially critical cellular pathways that control neuronal and vascular injury. In particular, previously unrecognized cellular mechanisms that are endogenous to the brain, but may have been considered without close association to the nervous system may offer the most novel and potent therapeutic strategies.

Given this premise, the protein erythropoietin (EPO), well known as a mediator of erythroid maturation in the hematopoietic system, but with exogenous expression in the brain, may represent a prime therapeutic candidate for the treatment of neuronal and vascular injury. Initially considered to primarily mediate the proliferation and differentiation of erythroid progenitors, EPO has emerged as a versatile growth factor that may play a significant role in the nervous system. EPO was the first cloned hematopoietic growth factor. It is a low molecular weight (30 kDa) glycoprotein that is produced in the fetal liver and subsequently in the adult kidney (Schuster et al., 1992). The primary function of EPO, which is fostered by the activation of the EPO receptor (EPOR) and subsequent signal transduction pathways, is to promote proliferation, differentiation, and

survival of erythroid progenitors resulting in the increased production of red blood cells. Erythropoiesis was considered to be the sole physiological action of EPO until EPO and the EPOR were found to be expressed in other organs outside of the liver and the kidney. As a result, the function of EPO is known to extend beyond erythropoiesis. For example, EPO may play a role in the meditation of hypertension by elevating vascular resistance and complicating a patient's clinical course during chronic treatment for anemia (Adamson, 1989). In addition, the expression of EPO in the central nervous system (CNS) may suggest a potential function for this protein in the CNS. Both EPO and the erythropoietin receptor EPOR are expressed throughout the nervous system in neurons, endothelial cells, and astrocytes in the cerebral cortex, hippocampus, and the amygdala (Morishita et al., 1997; Nagai et al., 2001; Chong et al., 2002c) (Figure 1). In cellular injury paradigms, EPO has been shown to provide protection against toxic insults, such as ischemia and free radical injury (Bernaudin et al., 1999; Chong et al., 2002b; Wen et al., 2002). To further the development of EPO as a novel neuroprotectant against both acute and chronic neurodegenerative disease, it is first critical to understand the cellular pathways that may mediate neuronal injury and are subsequently susceptible to modulation by EPO. This chapter will focus on the role of EPO in both neuronal and vascular systems in the CNS as a potential therapeutic agent for acute and chronic degenerative diseases.

The molecular building blocks of EPO

The EPO protein is the encoding product of EPO gene. The human EPO gene was cloned in 1985. It is located on chromosome 7 and exists as a single copy in a 5.4 kb region of the genomic DNA. The EPO gene encodes a polypeptide chain containing 193 amino acids (Jacobs et al., 1985). A 27 amino acid hydrophobic secretory leader at the amino-terminal is cleaved during secretion of EPO yielding a 166 amino acid peptide. In addition, a carboxy-terminal arginine in position of 166 is also removed both in mature human and recombinant human EPO (rhEPO) (Imai et al., 1990). As a result, the circulatory mature protein of EPO is a 165 amino acid peptide.

There are two disulfide bonds formed between cysteines at positions 7 and 160 and at positions 29 and 33. The requirement of disulfide bridges was demonstrated by the evidence that the reduction of the bonds resulted in the loss of the biologic activity of EPO. Alkylation of the sulfhydryl groups results in irreversible loss of the biological activity of EPO. Re-oxidization of EPO after reduction by guanidine HCl leads to regeneration of 85% of its biological activity (Wang et al., 1985). Cysteine 33 replacement with proline also reduces the biological function of EPO. These results suggest that the two disulfide bridges are necessary for EPO function.

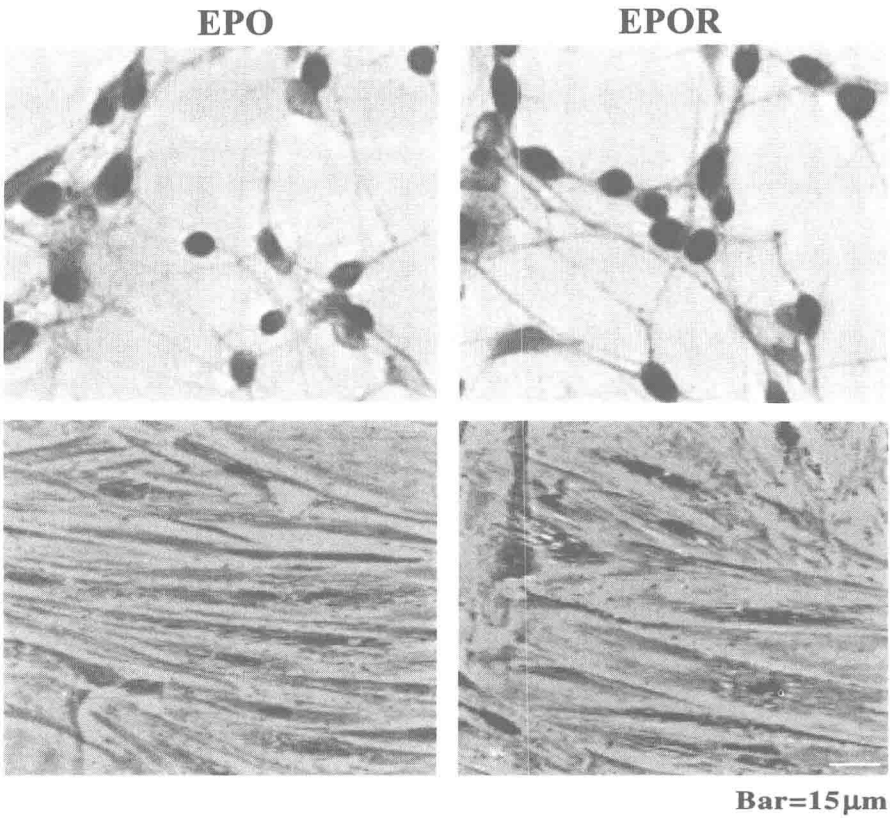

Figure 1. EPO and its receptor EPOR are constitutively expressed in rat hippocampal neurons and cerebral microvascular endothelial cells. Cell cultures were subjected to immunohistochemical detection for EPO and EPOR by using a rabbit primary polyclonal anti-EPO (1:1000) and anti-EPOR antibody (1:1000). Biotinylated horse anti-rabbit antibody was used as a secondary antibody (1:100). Representative pictures demonstrate that EPO and EPOR are expressed in hippocampal neurons (top panels) and cerebral microvascular endothelial cells (bottom panels).

EPO is a glycoprotien and the carbohydrate content contributes to almost 40% of its molecular weight. There are four glycosylated chains including three *N*-linked and one *O*-linked acidic oligosaccharide side chains. *N*-linked glycosylation sites are at the positions 24, 38, and 83 of aspartyl residues, while the *O*-linked glycosylation site is at position 126 (Seryl residues). Three *N*-glycan chains of human EPO consist of the tetra-antennary structure with or without N-acetyllactosamine repeating units (Tsuda et al., 1988). The *O*-linked sugar chain is composed of Gal-GalNAc and sialic acids (Sasaki et al., 1987).

The glycosylated chains are also important for the biological activity of EPO. Human EPO is stabilized by the carbohydrate chains (Toyoda et al.,

2000) and the oligosaccharides in EPO may protect the EPO protein structure from oxygen radical activity (Uchida et al., 1997). The N-glycosylated chains contribute to the thermal stability of EPO (Tsuda et al., 1988). In addition, the *N*- and *O*-linked chains may be necessary for the secretion of the mature EPO (Krantz, 1991). Replacement of asparagines 38 and 83 by glutamate or serine 126 by glycine can decrease the secretion of EPO (Dube et al., 1988). The presence of the carbohydrates also are important in the control of EPO metabolism, since EPO with high sialic acid content can be easily cleared by the body through specific binding in the liver (Tsuda et al., 1990).

Formation of EPO

EPO production is regulated by tissue oxygen supply. A deficiency in tissue oxygen results in EPO production not only in the kidney and liver (Jelkmann, 1992), but also in the brain (Marti et al., 1996). The hypoxia-dependent production of EPO in the kidney appears to be transient, while EPO production in the brain is more sustained (Chikuma et al., 2000). Additional studies in the brains of rodents and primates subjected to systemic hypoxia demonstrate an increase in production of EPO mRNA (Marti et al., 1996). Furthermore, neuronal cell lines have been found to retain the capacity to express the EPO gene in an oxygen-dependent manner (Stolze et al., 2002). Cerebral ischemia that leads to a deficiency of brain oxygen also can result in a significant increase in the expression of EPO and the EPOR in neurons, astrocytes, and cerebral microvascular endothelial cells (ECs) in mice (Bernaudin et al., 1999).

Hypoxia-inducible factor 1 (HIF-1) is essential for the production of EPO in response to hypoxia. Gene transcription of EPO is mediated by the transcription enhancer located in the 3 -flanking region of the EPO gene that specifically binds to HIF-1. HIF-1 is a basic helix-loop-helix heterodimeric transcription factor containing two subunits, HIF-1α and HIF-1β (Wang and Semenza, 1995). HIF-1β is a constitutively expressed, 91-94 kDa subunit that was characterized previously as aryl hydrocarbon receptor nuclear translocator (ARNT) (Hoffman, 1991). HIF-α is a 120 kDa, oxygen-labile subunit that undergoes rapid degradation via the ubiquitin-proteasome pathway under normoxic conditions (Huang et al., 1998). Upon hypoxia exposure, degradation of HIF-1α is impaired by blocking its association with von Hippel-Lindau protein that targets HIF-1α for proteasome (Maxwell et al., 1999). HIF-1α translocates to the nucleus and heterodimerizes with HIF-1β to form a stable HIF-1 complex. The HIF complex binds to the conserved sequence (5 RCGTG3) near the 5 end of the hypoxia-responsive enhancer of the EPO gene to up regulate EPO gene transcription (Bunn et al., 1998). Increased DNA binding activity of HIF-1 has been observed in rat cortical neurons during oxygen glucose deprivation and oxidative stress (Ruscher et al., 1998; Zaman et al., 1999) and in neuroblastoma cell lines during oxygen

stress (Halterman et al., 1999). These results suggest that HIF-1 may function as oxygen sensor regulating adaptive gene transcription and resulting in the production of EPO protein during hypoxia in the CNS.

The production of EPO in female reproductive organs is estrogen-dependent. Administration of 17β-estradiol (E_2), which controls the cyclic development of the uterine endometrium, can lead to a rapid and transient increase in EPO mRNA in the uterus (Yasuda et al., 1998). Hypoxia induced EPO mRNA expression in uterine tissue occurs only in the presence of E_2. This induction by hypoxia in the uterus is less pronounced than in the kidney (Chikuma et al., 2000). Oviduct and ovary production of EPO is also E_2 dependent (Masuda et al., 2000).

Erythropoiesis

EPO is the principal modulator of erythropoiesis. Yet, a diminished concentration of red blood cells is not the direct regulator of EPO production. Production of EPO and its potentiation of erythropoiesis are oxygen dependent. The plasma level of EPO is increased up to 1,000 fold above normal levels in response to hypoxia (Jelkmann, 1992). Circulating EPO binds to its receptor (EPOR) expressed on erythroid progenitors resulting in the stimulation of erythropoiesis. This subsequently leads to an elevation in the number of mature erythrocytes and the improvement of oxygen supply (Bauer, 1995). An impairment in EPO production as a consequence of renal failure results in the deficiency of circulating erythrocytes and severe anemia (Jelkmann, 1992). Since EPO functions as an erythropoietic factor, it has been widely used in the treatment of anemia (Eckardt, 2001).

Vascular control

Hypertension can complicate recombinant human EPO (rhEPO) during therapy for anemia (Adamson, 1989). Several mechanisms have been proposed to account for the elevation in vascular resistance and the subsequent development of high blood pressure during EPO chronic administration. Early studies recognized that increased blood viscosity as a result of rising hematocrit values contributed to high blood pressure during chronic treatment with EPO (Schaefer et al., 1988). The correction of anemia by EPO resulted in an increase in erythrocyte mass and blood viscosity (Steffen et al., 1989) and the reversal of hypoxic vasodilation in uremic anemia (Roger et al., 1992). Yet, further studies demonstrated that constant dosage and chronic administration of EPO in iron-deficient renal anemic patients did not increase blood pressure despite a dramatic increase in hematocrit by iron repletion (Kaupke et al., 1994). Thus, EPO can lead to