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FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2015/2016

Maria Francisca Torcato Ribeiro Dias de Freitas

Molecular mechanisms underlying hyperoxia-induced acute lung injury

março, 2016

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Mestrado Integrado em Medicina

Área: Fisiologia

Tipologia: Monografia

Trabalho efetuado sob a Orientação de:

Doutor Roberto Roncon-Albuquerque

Trabalho organizado de acordo com as normas da revista:

Respiratory Medicine

março, 2016

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FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR
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NOME

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NÚMERO DE ESTUDANTE	DATA DE CONCLUSÃO
200907007	

DESIGNAÇÃO DA ÁREA DO PROJECTO

FISIOLOGIA

TÍTULO ~~DISSERTAÇÃO~~/MONOGRAFIA (riscar o que não interessa)

MOLECULAR MECHANISMS UNDERLYING HYPEROXIA-INDUCED ACUTE LUNG INJURY

ORIENTADOR

DR. ROBERTO RONCON-ALBUQUERQUE

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Aos os meus pais, irmãos e

Ricardo pelo apoio incondicional

Molecular mechanisms underlying hyperoxia-induced acute lung injury

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ABSTRACT

The management of acute hypoxemic respiratory failure frequently includes the use of supraphysiological fractions of inspired oxygen (FiO_2), which can be beneficial in the short-term but not without risks in the long-term causing acute lung injury (ALI). Over the last few years much attention has been focused on the intracellular signalling transduction pathways that lead to hyperoxia-induced cell damage, particularly MAP kinase cascades. Identification of involved signalling molecules and understanding the regulation of the main signal transduction pathways might provide the basis for improving the outcome of the patients under high FiO_2 through more effective therapeutic interventions. This review, which includes studies published from 1987 to 2015, presents an overview on recent progresses in the hyperoxia ALI field with special emphasis to potential therapeutic targets and clinical approaches based on the molecular mechanisms underlying hyperoxia-induced inflammation. Further studies are needed to gain deeper insight into controversial molecular mechanisms underlying hyperoxia-induced cell death, which may play a critical role in future pharmacological interventions, as well as into hyperoxia-induced cell damage, that could monitor and therefore prevent hyperoxia ALI.

Keywords: acute lung injury; fraction of inspired oxygen; hyperoxia; MAP kinase.

INTRODUCTION

The management of acute hypoxemic respiratory failure frequently includes the use of supraphysiological oxygen concentrations in inspired air (pO_2 (a) > 300 mmHg) to ensure adequate blood oxygenation. [1, 2] Although potentially lifesaving in the short-term, prolonged hyperoxia is not without risks and has been implicated in organ toxicity such as ALI. [3-5] On the one hand, O_2 plays a vital role in ATP synthesis, on the other hand it is responsible for the production of ROS capable of damaging alveolar epithelial cells causing disturbances in pulmonary system and gas exchange impairment. There is a narrow margin between therapeutic and deleterious effects of high concentrations of inspired oxygen suggesting that the potential benefits and risks of ventilation with high FiO_2 (0.8–1.0) must be weighed since the question whether exposure to supranormal PaO_2 is safe in critically ill patients remains unanswered.

PMNs are the major inflammatory cells involved in the process of ALI, generating ROS, leading to alveolar epithelial cells death. [6-9] ROS have been shown to participate in the pathogenesis of many human diseases; however, the biochemical mechanisms by which ROS cause cell damage and ultimately organ dysfunction are not completely understood. [1, 2, 10, 11] Under hyperoxic conditions, excessive ROS act as direct cell toxins as well as secondary messengers by inducing the activation of intracellular transduction pathways and secretion of proinflammatory cytokines by the alveolar epithelial cells. [12] This leads to destruction of the alveolar-capillary barrier, increased pulmonary permeability, endothelial and epithelial cell death and PMNs influx into alveolar spaces. [12-15]

Abbreviations: ALI (acute lung injury); AP-1 (activator protein 1); ATP (adenosine triphosphate); CHI3L (chitinase 3-like 1); DPI (diphenyleneiodonium); DUOX (dual oxidase); Egr-1 (early growth response gene-1); ENac (epithelial sodium channel); EP (ethylpyruvate); ERK (extracellular signal-regulated kinase); HMGB-1 (high mobility group box-1); HS (hydrogen-rich saline); JNK (c-Jun N-terminal kinase); LC3B (protein 1 light chain 3B); MAPK (MAP kinase); MIP-2 (macrophage inflammatory protein-2); mtALDH (mitochondrial aldeyde dehydrogenase); NF-Kb (nuclear factor Kb); NOX (NADPH oxidase); Nrf2 (nuclear fator-like 2); PAI-1 (plasminogen activator inhibitor-1); ROS (reactive oxygen species); PKCδ (protein Kinase Cδ); PMNs (polymorphonuclear leucocytes); RA (retinoic acid); SP (substance P); TGFβ1 (transforming growth-factor beta-1); TNF-α (tumor necrosis factor alpha); Trx (thioredoxin).

Over the last few years much attention has been focused on the signalling pathways and proinflammatory cytokines that lead to hyperoxia-exposed cells death during supplemental oxygen therapy. [16, 17] The comprehension of the molecular mechanisms responsible for the development of ALI is based on the identification of signaling molecules that are crucial in response to lung injury. A thorough understanding into the regulation of the main signal transduction pathways that can lead to alveolar epithelial cell injury after prolonged hyperoxia might provide the basis for improving the outcome of the patients under high FiO_2 through more effective therapeutic interventions.

The purpose of this review is to provide a critical evaluation of the recent bibliography on hyperoxia ALI, focusing on recently described intracellular molecular pathways and exploring potential therapeutic targets and future clinical applications.

METHODS

Eligible studies were identified by an electronic search of PubMed, involving studies published from 1987 to 2015. The sensitive search strategy combined the following keywords: *molecular mechanisms; hyperoxia;* and *acute lung injury*. All articles and cross-referenced studies from retrieved articles were screened for pertinent information and reviewed by both Authors. Inclusion criteria consisted in experimental and systematic review articles, published as original studies, with available abstract. Publications not written in English were excluded.

SIGNAL TRANSDUCTION PATHWAYS

ROS generated by NOX family enzymes play a pivotal role in hyperoxia-induced ALI. [2, 13] Although the effect of ROS in ALI has not been completely defined, previous studies demonstrated direct pulmonary cells injury through lipid peroxidation, enzyme inactivation, DNA damage, and cellular reducing agents decrease. [18] Distinct NOX enzymes, may specifically activate a range of different signaling pathways by modulating their subcellular location, expression level and interaction with membrane proteins. [18-22] However, previous studies have reported NOX1 as the main participant member of NOX family in ALI by stimulating ROS generation and cell death in lung epithelium and endothelium, whether NOX2 and NOX4 induce cell migration and cell death, although controversy exists. [22-24]

DUOXs are major NOX homologues and a significant source of ROS in lung epithelium. [2] Previous studies have suggested that DUOX-generated ROS play a central role in the regulation of innate immune responses and modulation of cell death in airway epithelial cells exposed to prolonged hyperoxia, rather than NOX1. [2, 18, 25]

Generation of ROS under supraphysiological concentrations of oxygen is reported to be one of the main damaging stimuli that can induce or mediate phosphorylation of MAPK signal transduction pathways by altering the structure and function of signaling proteins through modification of critical aminoacid residues. [19, 26, 27] MAPK signal transduction pathways regulate a wide range of vital cellular mechanisms, such as cell growth, proliferation, differentiation, stress responses, and ultimately survival and apoptosis through modulation of distinct intracellular and nuclear substrates. [28, 29] To date, several studies described four distinct MAPK cascades: ERK 1/2, JNK, p38 and ERK 5. [28]

Hyperoxia-induced ROS, together with NOX1 activation, are responsible for the triggering of ERK 1/2 pathway characterized by the activation of a wide range of substrates in distinct locations, particularly the nucleus (fig. 1). [28, 30] In addition to direct interaction between ROS and ERK 1/2, ROS are responsible for decreasing mtALDH activity and stimulating CHI3L. [28] As a defense mechanism against the damaging effects induced by hyperoxia, phosphorylated ERK 1/2 pathway promotes survival genes transcription, such as Egr-1, Nrf2, and AP-1. [28] Between the survival genes transcripted in the ERK 1/2 signaling pathway, previous studies demonstrated that decreased transcription of Nrf2 promotes increased ALI and mortality through suppressed expression of several antioxidant enzymes and superoxide dismutase, which are involved in cellular protection against hyperoxia-induced inflammation. [2, 20, 30] Although most studies describe ERK 1/2 cascade as pro-survival signal transduction pathway in hyperoxia-exposed cells, additional reports have mentioned alveolar macrophage apoptosis under other conditions that can be ascribed to the specific cell type involved. [10, 27] Thus, in hyperoxic conditions, ERK 1/2 phosphorylation might have a dual role as either promoter or inhibitor of apoptosis, and these roles appear depend on the cell type and culture conditions. [31]

As a key member of the MAPK family, JNK activation in hyperoxia-exposed cells is induced by ROS and NOX1 (fig. 2). [19] Once activated, JNK induces phosphorylation of several substrates located in the cytoplasm and nucleus promoting transcription of apoptotic genes as well as immune and stress responses. [28] Several studies have reported AP-1 complex as a major target of phosphorylated JNK. [28] Activated in several mechanisms, including oxidant signaling, immune responses, and cell differentiation and apoptosis, AP-1 complex has been shown to play an important role in modulating both cell proliferation and death in a cell-type and stimulus-

dependent manner. [29, 32] However, Romashko et al. demonstrated that sustained activation of AP-1 is more closely associated with apoptosis. [32] Together with AP-1 complex, TGFβ1 is involved in inflammation and hyperoxia-induced cell death as well as impaired alveolarization. [33] Although the final effect of JNK activation supports necrosis and apoptosis, Porzionato et al. suggested that JNK phosphorylation might stimulate pro-survival mediators such as microtubule-associated LC3B. [28] Thus, in hyperoxia-exposed cells, the effect of JNK pathway activation is distinct, depending on the stimuli and strength as well as duration of phosphorylation, and can range from apoptosis to increased survival. [28, 34] Most studies propose JNK as a pro-apoptotic factor; however a protective role of JNK activation in response to hyperoxia has already been described. [35]

Similar to other members of MAPK family, p38 activation caused by stress stimuli and signals transmitted through the recruitment of specific receptors, plays an important role in hyperoxia-induced lung injury (fig. 3). [28, 32] Li et al. reported p38 has a central regulator of immunological effects, cell apoptosis, senescence, and survival as well as cell-cycle checkpoints. [34] Apart from direct activation from ROS, p38 activity is stimulated by ROS-induced PKCδ and CH3IL. [28] To date, p38 activation role in ALI is variable. Further analysis will be needed to identify the contributing factors for the variability of p38 activation under hyperoxic conditions. The role of p38 activation in hyperoxia-exposed cell and consequent lung damage is yet unclear, while some reports have described protective effects, most studies have reported detrimental actions. [36, 37]

Under hyperoxic conditions, ROS-mediated cell death is partially dependent on caspase-mediated signaling pathways (fig. 4). [19, 29] Hyperoxia-induced activation of signal transduction pathways leads to either necrosis or apoptosis. [14, 38, 39] Indeed,

both mechanisms can co-exist, once they share similar induction agents in similar cell types. [32, 38] ROS induce death receptor Fas activation with resultant stimulation of initiator caspase 8. [10] This triggers a range of events as activation of Bax, Bid, Bim and Back which increase PKCδ expression. [10] As an apoptotic modulator, PKCδ stimulates cleavage of executioner caspases 3 and 9, culminating in cell death by apoptosis and/or necrosis. [10] Caspase 3 is considered the most important of the effector caspases. [40] The main role of apoptosis in epithelial damage under hyperoxic conditions remains unclear, however several mechanisms underlying hyperoxia-induced cell death have already been proposed. [41]

Pro-inflammatory cytokines have been implicated in mediating neutrophil influx into hyperoxic lungs; however, molecular processes underlying PMNs recruitment remain unclear. [10, 40] Such pro-inflammatory cytokines, including TNF-α, MIP-2, PAI-1, IL-1β, IL-6 and IL-8, are crucial mediators in the early stages of inflammatory response. [1, 42] The transcriptional factor NF-κB is often described as the factor required for maximal expression of numerous cytokines implicated in the HALI. [32, 40] Entezari et al. determined that hyperoxia-exposed cell injury is characterized by increased extracellular HMGB-1 production. [9] A DNA-binding protein, extracellular HMGB-1 triggers an overwhelming late inflammatory response that promotes the progression of ALI. [43-46] Indeed, extracellular HMGB-1 promotes the release of cytokines such has IL-1β, TNF-α, MIP-2 and macrophage migration inhibitory factor, and conversely, cytokines control further release of HMGB-1 to both plasma and lung epithelial lining fluids. [9]

POTENTIAL THERAPEUTIC TARGETS

Understanding the molecular mechanisms may be useful for the identification of potential therapeutic targets as well as biomarkers to be monitored in the course of HALI (Table 1).

Regarding the described role of DUOX 2 in lung injury after prolonged hyperoxia, Kim et al. reported that an acute reduction of DUOX 2 expression is sufficient to inhibit ALI and consequent cell death. [2] Simultaneously, Xu et al. demonstrated that the overexpression of mtALDH attenuates hyperoxia-induced cell death by inducing ERK 1/2 phosphorylation, and inhibition of ERK 1/2 cascade partially suppresses the positive effect of mtALDH, reporting ERK 1/2 activation through mtALDH as a defense mechanism against hyperoxia. [47] As well as mtALDH overexpression, CHI3L knock-down, and increased survival genes transcription has been shown to play a protective role from hyperoxia-induced apoptosis by increasing ERK 1/2 phosphorylation. [28, 48] In addition, Kim et al. demonstrated increased survival in CHI3L-deficient cells through p38 attenuation. [48] These findings may prove helpful in developing potential therapies based on DUOX 2 specific inhibitors in the treatment of ALI.

JNK cascade exposure to supranormal concentrations of O_2 activates morphological and biochemical markers, such as LC3B. [49] Tanaka et al. reported that LC3B overexpression confers cytoprotection against hyperoxia injury through autophagy stimulation and inhibition of caspase-3 cleavage. [28, 49] Meanwhile, several studies described the selective inhibition of p38 as a sufficient mechanism to increase cell survival in hyperoxic-exposed cells. [36, 37]

CLINICAL IMPLICATIONS

Over the last few years, much progress has been made in developing possible therapeutic strategies involved in hyperoxia-induced ALI (table 2). Regarding the described role of NOX enzymes, their inhibition may have significant effect in improving therapies to alleviate hyperoxia-induced lung damage through suppression of endothelial and epithelial cell death. Papaiahgari et al. demonstrated that inhibition of NOX by DPI considerably reduces the generation of intracellular and extracellular ROS, suggesting that NOX actively contributes to the conversion of oxygen into O_2^- and its inhibition may be helpful as a potential therapeutic target for ALI by reducing oxidative stress induced by hyperoxia. [2, 30]

Porzionato et al. have identified numerous exogenous stimulators of MAPK family members as protective modulators of hyperoxic stimuli. [28] Incubation or administration of hyperoxia-exposed cells with ATP, inosine and laminin substrates results in increased survival cell response through ERK 1/2 phosphorylation. [28] Ahmad et al. demonstrated that ATP release and subsequent ATP-mediated signaling events are vital for cell survival in hyperoxia. [50] In addition, Buckley et al. described the protective role of inosine treatment during hyperoxic exposure. [51] Meanwhile, Buckley et al. also demonstrated that culture of cells on laminin substrates, with respect to other plastic supports, resulted in increased phosphorylation of ERK 1/2. [52] Huan et al. added that the addition of SP to cell cultures can promote proliferation and inhibit apoptosis by suppressing JNK and p38 signal pathways after hyperoxia exposure, which attenuates induced oxidative lung injury. [53, 54] In addition, Li et al. and Chen et al. reported ERK 1/2, JNK and p38 significant increase under prolonged hyperoxic conditions. [55, 56] Additional studies demonstrated that RA and Trx treatment induced JNK and p38 decline and ERK 1/2 further elevation. [55, 56]

Sureshbabu et al. have reported TGFβ1 as a critical mediator of ALI. [33] Indeed, Tamarapu et al. described TGFβ1 as a modulator of ENac that reduces its expression, and alveolar sodium transport in epithelial cells. [57] The inhibitory effect of TGFβ1 on ENac, the main determinant of alveolar fluid clearance across the alveolar epithelium, is mediated by ERK 1/2 cascade activation. [57] The same study has identified serotonin as an endogenous inhibitor of ENac through TGFβ1 expression stimulation. [57] Therefore, miR-16, as a molecule that regulates serotonin system and upregulates ENac should be considered as a potential therapeutic approach to modulate ENac expression and restore alveolar fluid balance in ALI. [57]

Regarding p38 cascade, once activated by oxidative stress, PKCδ has been reported as an important apoptosis modulator through increased production of the caspase-induced PKCδ cleavage products. [28] Interestingly, Grinnell et al. demonstrated that PKCδ chemical inhibitor (rottlerin) significantly attenuated p38 activation as well as apoptosis, suggesting a potential dual role for PKCδ in ROS-induced apoptosis. [28, 58] As mentioned, rottlerin acts as an upstream regulator of p38 activation and as an inducer of DNA damage, through its caspase-3-dependent cleavage fragment, what may prove helpful in developing future therapies based on PKCδ chemical inhibitors in the treatment of ALI. [58] Simultaneously, Otterbein et al. reported the protective role of low concentration CO in hyperoxic lung injury, extending survival and exerting potent anti-inflammatory effects with reduced inflammatory cell influx into the lungs and marked attenuation in the expression of pro-inflammatory cytokines. [59] Indeed, exogenous administration of CO limits the progress of histopathological changes and attenuates cytokine expression induced by hyperoxia. [28, 59]

Xie et al. demonstrated decreased caspase 3 activity after H_2 or HS treatment, suggesting a preventing role of H_2 treatment in lung cell apoptosis. [40] The same study added that the effective therapeutic role of H_2 or HS in hyperoxia-induced ALI also occurred through downregulation of inflammation and apoptosis via suppressing NF-κB activation. [40] Most of the data suggested endogenous NO, similarly to H_2, exerts an effective therapeutic role in many disorders including oxygen toxicity through decreasing oxidative stress, inflammation, and apoptosis, although controversy exists. [10, 40]

Entezari et al. suggested a link between extracellular HMGB-1 and ALI pathogenesis once low levels of extracellular HMGB-1 decreased inflammation. [9] In addition, they have shown that inhibition of HMGB-1 by neutralizing anti-HMGB-1 antibodies and small molecule EP significantly protects lung tissue against hyperoxia-induced extracellular HMGB-1. [9] In addition, heparin was shown to modulate infiltration of neutrophils and improve gas exchange. [1] Thus, Li et al. demonstrated that pharmacological inhibition with enoxaparine reduced HMGB-1 and PAI-1 production during prolonged hyperoxia. [1]

CONCLUSION

The present review focused on recent progresses in the ALI field with special emphasis to molecular mechanisms underlying hyperoxia-induced inflammation after high FiO_2 ventilation. The wide involvement of signal transduction pathways in lung responses to hyperoxia suggests their modulation may have significant effect in improving therapies to alleviate ALI in patients on oxygen therapy through suppression of endothelial and epithelial cell death. In summary, further analysis is needed to gain deeper insight into controversial molecular mechanisms underlying hyperoxia-induced cell death which may play critical role in future pharmaceutical interventions as well as biomarkers monitoring targeted at prevention or resolution of ALI.

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Table 1. Overview of therapeutic targets in hyperoxia-induced ALI.

Reference	Year	Model	Molecule	Mechanism	Role in ALI
[2]	2014	Mice	DUOX 2	ERK 1/2 and JNK phosphorylation	Deleterious
[28]	2015	Mice	Survival genes	ERK 1/2 phosphorylation	Protector
[47]	2006	Mice	mtALDH	ERK 1/2 phosphorylation	Protector
[48]	2012	Mice	CH3IL	ERK 1/2 phosphorylation and p38 inhibition	Deleterious
[49]	2012	Mice	LC3B	autophagy stimulation and caspase-3 cleavage inhibition	Protector

Table 2. Overview of potential pharmacological treatments oh hyperoxia-induced ALI.

Reference	Year	Model	Mechanism	Future therapy
[1]	2011	Mice	PAI-1 and HMGB-1 inhibition	Enoxaparin
[9]	2014	Mice	HMGB-1 inhibition	Anti-HMGB-1 and EP
[40]	2012	Mice	Caspase 3/9 and Nf κ B inhibition	H_2 and HS
[50]	2004	Mice	ERK 1/2 phosphorylation	ATP
[51]	2005	Mice	ERK 1/2 phosphorylation	Inosine
[52]	1999	Mice	ERK 1/2 phosphorylation	Laminin substrates
[53]	2009	Mice	JNK and p38 inhibition	SP
[55]	2006	Mice	ERK 1/2 phosphorylation JNK and p38 inhibition	RA
[56]	2010	Mice	JNK and p38 inhibition	Trx
[59]	2015	Mice	p38 inhibition	CO
[30]	2004	Mice	NOX inhibition	DPI
[58]	2012	Mice	PKC δ inhibition	rottlerin
[57]	2012	Mice	TGF β 1 inhibition	miR-16

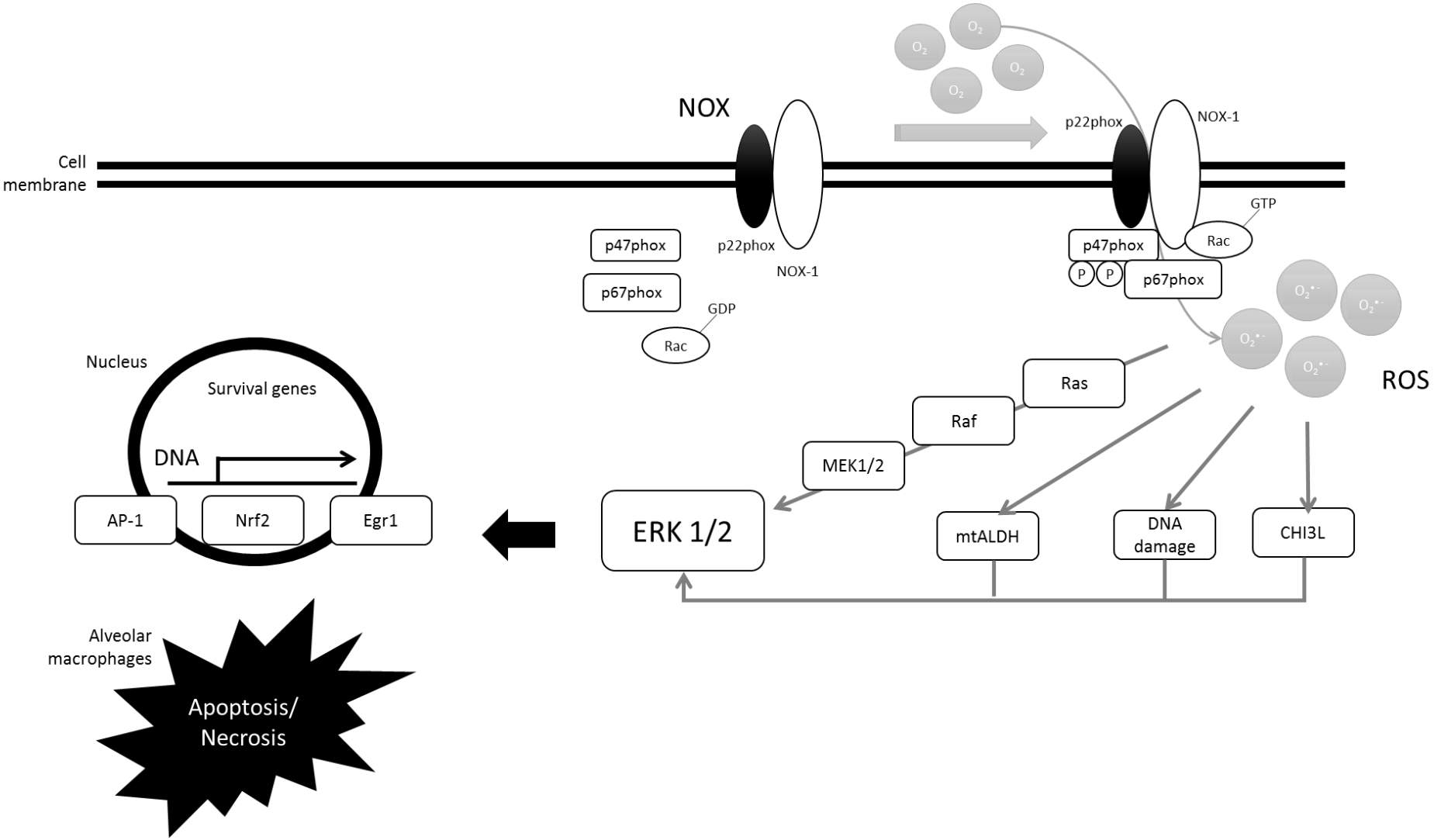
FIGURE LEGENDS:

Figure 1. Schematic diagram of the extracelular signal-regulated kinase (ERK) signaling pathway in lung cells exposed to prolonged hyperoxia: supraphysiological oxygen concentrations in inspired air can lead to reactive oxygen species (ROS) production via NADPH oxidase (NOX) phosphorylation. ROS promote stimulation of mitochondrial aldeyde dehydrogenase (mtALDH) and chitinase 3-like 1 (CHI3L), and cause DNA damage. The signal is also transduced to small GTP-binding proteins (Ras), which in turn activate the core unit of the cascade composed of a MAPKKK (Raf), a MAPKK (MEK1/2), and MAPK (Erk). Activated ERK leads to transcription of survival genes, like activator protein 1 (AP-1), early growth response gene-1 (Egr-1), nuclear factor-like 2 (Nrf2), which inhibit necrotic and apoptotic cell death. In contrast, ERK activation might induce apoptosis/necrosis of alveolar macrophages.

Figure 2. Schematic diagram of the c-Jun N-terminal kinase (JNK) signaling pathway in lung cells exposed to prolonged hyperoxia: supraphysiological oxygen concentrations in inspired air can lead to reactive oxygen species (ROS) production via NADPH oxidase (NOX) phosphorylation. ROS stimulate JNK activation through the membrane proximal kinase MAPKKK, typically MEKK1–4, that phosphorylates and activates MKK4 or MKK7, the JNK kinases. Once phosphorylated, JNK promotes inflammation and impaired alveolarization through induction of activator protein 1 (AP-1) and transforming growth factor-beta 1 (TGFβ1), respectively. Thus, the final effect of JNK activation supports necrosis and apoptosis. JNK might stimulate microtubule associated protein 1 light chain 3B (LC3B) factor, which would induce autophagic mechanisms to protect the cell against hyperoxia.

Figure 3. Schematic diagram of the p38 signaling pathway in lung cells exposed to prolonged hyperoxia: supraphysiological oxygen concentrations in inspired air can lead to reactive oxygen species (ROS) production, via NADPH oxidase (NOX) phosphorylation. ROS induce p38 activation through protein kinase Cδ (PKCδ), chitinase 3-like 1 (CHI3L), and also MAPKKK, typically MEKK 1 to 4 or a mixed lineage kinase (MLK) 2 or 3, that phosphorylate and activate MKK3 or 6, the p38 MAPK kinases. Once activated, p38 supports necrosis and apoptosis.

Figure 4. Schematic diagram of the apoptosis/necrosis signaling pathway in lung cells exposed to prolonged hyperoxia: supraphysiological oxygen concentrations in inspired air can lead to Fas receptor phosphorylation and further activation of caspase 8, which induces the activation of the pro-apoptotic proteins Bax, Bid, Bim and Back, resulting in increased protein kinase Cδ (PKCδ) expression. Once activated, PKCδ stimulates caspases 3 and 9, culminating in cell death by necrosis or apoptosis.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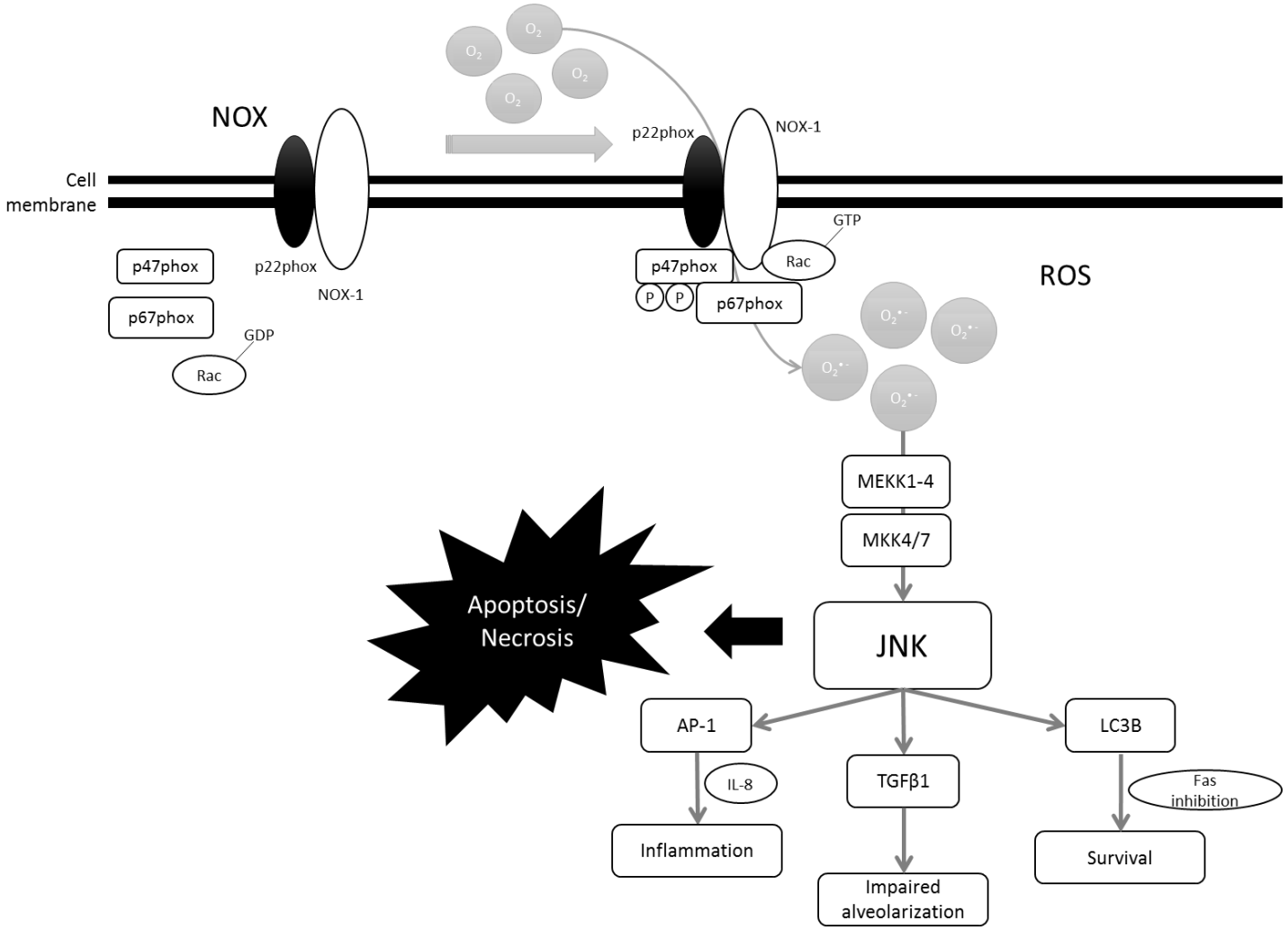
Apoptosis/ Necrosis

Figure 1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Survival

Figure 2

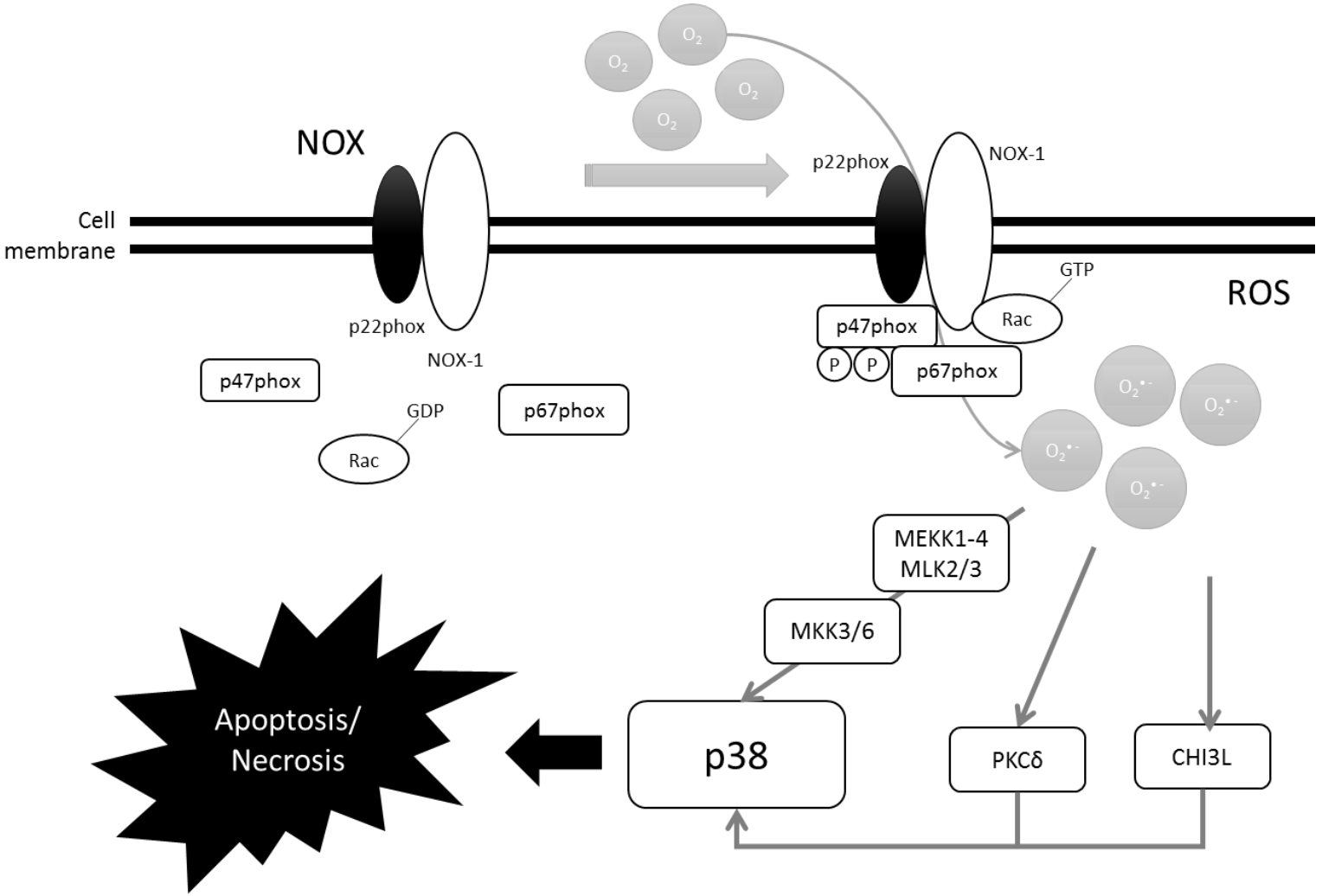
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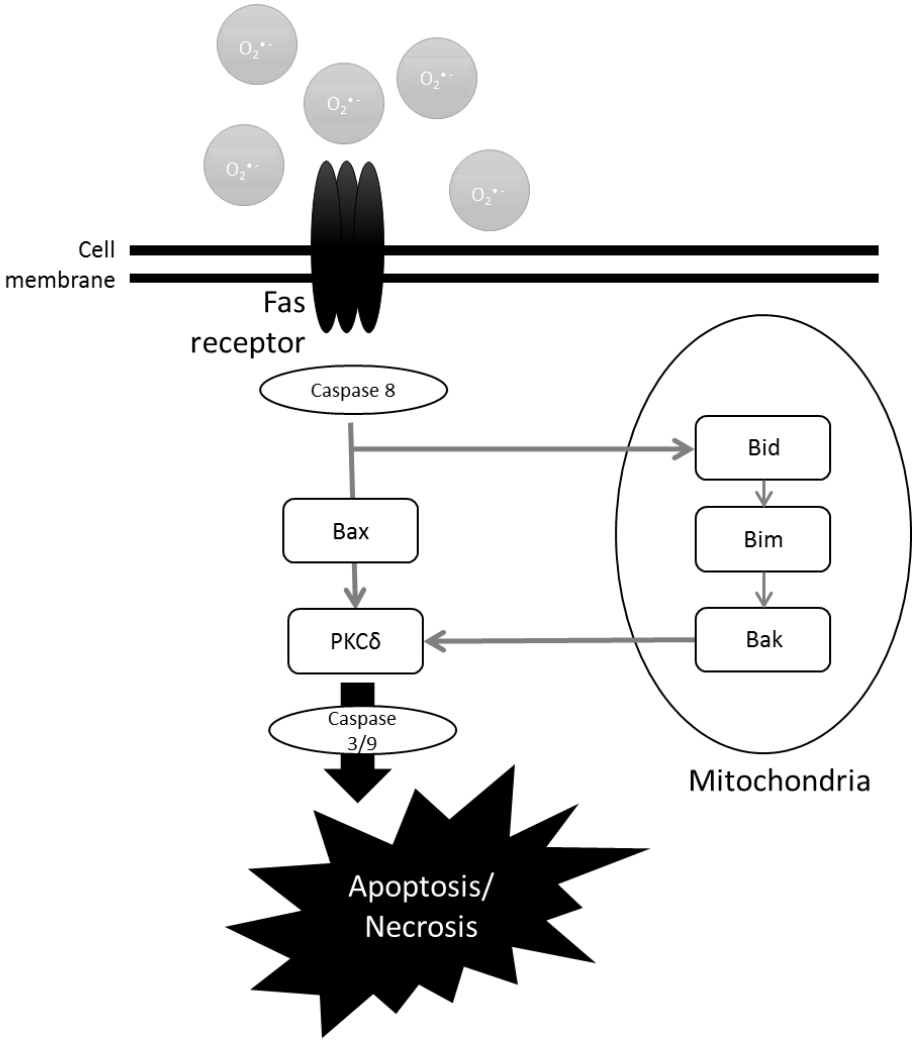
Figure 3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Caspase 8
Bid
Bax
Bim
PKCδ
Bak
Caspase 3/9
Mitochondria
Apoptosis/ Necrosis

Figure 4

Agradecimentos

Ao Doutor Roberto Roncon-Albuquerque, orientador desta Tese de Mestrado, os meus agradecimentos pelo rigor científico, disponibilidade e incentivo constante que me transmitiu na supervisão deste trabalho.

Aos meus pais, pela confiança e compreensão, agradeço o apoio constante nas fases boas e menos boas do meu percurso académico e pessoal, os valores que me transmitiram e por terem tornado este momento possível.

Aos meus irmãos, Luís, Joana e Fernando, que sempre me fizeram sentir capaz, obrigada pela paciência e compreensão.

Ao Ricardo, pelo apoio e força transmitida, amor e carinho demostrados, por ser um ouvinte paciente de todas as minhas dúvidas, inquietações e desânimos, muito obrigada.

À Mafalda, pela sua incansável amizade e apoio a qualquer hora e momento, obrigada.

ANEXOS

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Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available on our website:
https://www.elsevier.com/artworkinstructions.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.

• Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please see https://www.elsevier.com/artworkinstructions.

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Figure captions
Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

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As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

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Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

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One author has been designated as the corresponding author with contact details:

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- Full postal address

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- Keywords
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- All tables (including title, description, footnotes)

Further considerations

- Manuscript has been 'spell-checked' and 'grammar-checked'
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)

Printed version of figures (if applicable) in color or black-and-white

- Indicate clearly whether or not color or black-and-white in print is required.

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