Genetic Models in Applied Physiology
Invited Review: Effect of oxygen deprivation on cell cycle activity: a profile of delay and arrest

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Douglas, R. M., and G. G. Haddad. Invited Review: Effect of oxygen deprivation on cell cycle activity: a profile of delay and arrest. J Appl Physiol 94: 2068–2083, 2003;10.1152/japplphysiol.00037.2003.—One of the most fascinating fields that have emanated in the past few decades is developmental biology. This is not only the case from a research point of view but also from the angle of clinical care and treatment strategies. It is now well demonstrated that there are many diseases (some believe all diseases) that have their roots in embryogenesis or in early life, where nature and environment often team up to facilitate the genesis of disease. There is probably no better example to illustrate the interactions between nature and environment than in early life, as early as in the first several cell cycles. As will be apparent in this review, the cell cycle is a very regulated activity and this regulation is genetic in nature, with checkpoint proteins playing an important role in controlling the timing, the size, and the growth of daughter cells. However, it is also very clear, as will be discussed in this work, that the microenvironment of the first dividing cells is so important for the outcome of the organism. In this review, we will focus on the effect of one stress, that of hypoxia, on the young embryo and its cell division and growth. We will first review some of the cell cycle definitions and stages and then review briefly our current knowledge and its gaps in this area.

animal models; anoxia; cell division; cancer

ENVIRONMENTAL STRESSES can have direct effects on cell cycle activity. Ultraviolet (UV) and ionizing (IR) radiation can cause DNA damage that leads to dysplasia or even morbidity and mortality of the embryo (138). Other factors such as heat, overcrowding (contact inhibition), and dessication can lead to cell cycle arrest, resulting in either temporary suspended animation or eventual cell death. Oxygen (O2) deprivation or hypoxia is another such stress that appears to have a direct effect on cell cycle activity.

O2 deprivation is not only important in pathogenetic mechanisms of disease processes but is also involved in development and cell growth in early life, especially during embryogenesis (59, 159, 160). To illustrate the former, consider some of the disease conditions that we regularly encounter or some of the natural physiological stresses that are often imposed on organisms. A critical component of respiratory and cardiovascular diseases in children and adults as well as life at high altitude is the exposure to periodic or continuous episodes of hypoxia. These episodes, whether acute or chronic, can result in pulmonary hypertension and neurological and cardiovascular disorders (72, 95, 178, 179). Prolonged ischemia or hypoxia in fetuses or in neonates can cause irreversible brain damage and injury to O2-sensitive organs such as the lung, heart, and kidney (22, 79, 132, 166). Although such conditions are not clinically infrequent, the cellular and molecular events that take place in and outside cells are not well understood, mainly due to a lack of a suitable animal model.

Mammals are normally extremely intolerant of severe or prolonged hypoxic exposure, and O2 deprivation in utero can often ensue in fetal demise. It has also been demonstrated clinically that patients who live for prolonged periods of time with desaturated hemoglobin (such as with congenital heart disease) show stunted growth and development in early life (73) that is not likely related to dietary intake. Similar growth-related
problems have also been discovered for high-altitude sojourners (87). Therefore, low-\(O_2\) conditions may not only lead to injury but may also affect growth rate early in life.

However, several species, such as the turtle and brine shrimp, demonstrate imperviousness to lack of \(O_2\) (26, 88). We and others have previously shown that the fruit fly, \textit{Drosophila melanogaster} (\textit{D. melanogaster}), embryo or adult is also capable of surviving and recovering from several hours of prolonged hypoxia (42, 74, 176). The understanding of the cellular and molecular mechanisms underlying the response to hypoxia in this and other species has been essentially limited. It has been reported that hypoxia can lead to either hypoplasia as seen in the growth retardation reported in response to in utero hypoxia or hyperplasia as demonstrated in the pulmonary vasculature. How such a stress can produce contrasting effects is still under intensive experimentation, and the molecular mechanisms that permit these clearly varied responses to lack of \(O_2\) are not completely understood but are also being aggressively investigated. This review will address the advances that have been made in our understanding of the cellular hypoxia response in the recent past.

**THE CELL CYCLE**

An understanding of the regulation of the cell cycle is requisite in the attempt to determine how hypoxia might affect cell cycle activity. The mechanism of cellular duplication and replication is regulated by a complex hierarchy of genetic and metabolic networks. In the early 1970s, a rash of activity led to the elucidation of the essential underpinnings of the cell cycle machine, i.e., the oscillation of certain cell cycle proteins, namely the cyclins and their partners, the cyclin-dependent kinases (cdks), in a phasic, cyclical fashion (55). Cell cycle periodicity can also be viewed as linear sequential events that are iterated as needed by the organism (137). Rapid iterations of cell cycle activity occur in the early embryo and may be as short as 10 min per cycle. On the other hand, cell cycle length within individual tissues can be as long as 48 h per cycle, depending on the species and the developmental stage. Finally, a critical component of the cell cycle is the ability to cease replicative activity and to become quiescent. Cells can then reenter the cell cycle if the proper stimulus is provided or replication becomes deranged, as in cancer. Control of the cell cycle therefore involves modulation of several transition states and stages of varied length. The traditionally defined stages of the cell cycle are 1) \(S\) phase, during which DNA replication occurs; 2) gap phase 2 (G2), during which proteins required for mitosis are accumulated; 3) M phase (mitosis or maturation stage), during which chromatin condensation, nuclear envelope breakdown (NEBD), chromatid separation, and cytokinesis occur; 4) gap phase 1 (G1), during which genes necessary for DNA replication are activated and the protein agents of S phase progression are accumulated; and 5) G0, during which cells can exit the cell cycle and enter a state of differentiation or quiescence (Fig. 1).

Control of cell cycle activity can be exerted both during the various stages of the cell cycle and at the points of transition from state to state. Thus cell cycle activity can also be modulated at the G1/S and G2/M transition points. Environmental cues can also impact on cell cycle activity so that there are both endogenous and exogenous cell cycle regulatory factors that can modulate both the rate of cell cycle activity and the stage in which the cell must reside. External/extracellular modulation of cell cycle activity involves both mitogenic and pathological signals. Therefore, there are also intrinsic and extrinsic conditions that can result in reentry into the cell cycle or exit from the cycle, i.e., arrest or quiescence. Some of these extrinsic factors include contact inhibition, nutrient supply, cell size, temperature, and a variety of stresses such as hypoxia. We are particularly interested in the effect of environmental hypoxia on cell cycle activity and cell injury.

![Fig. 1. Analog diagram representing a simplified version of the cell cycle, with a focus on the G1/S phase transition. A minimum number of regulatory cell cycle proteins are depicted in this figure. Cyclin D/cyclin-dependent kinase (cdk)/4.6 is activated to initiate induction of S phase before cyclin E; however, both complexes are required. Cyclin D, in cooperation with cyclin E/cdk2, hyperphosphorylates retinoblastoma protein family (RBF), causing the release of RBF from the dimer elongation factor (E2F) dimerization partner (Dp), a transcription factor that activates several S-phase-requiring genes. Cyclin A/cdk2 also participates in progress through S phase. In response to DNA damage, several regulatory pathways are activated to prevent entry into S phase. Ataxia telangiectasia mutated (ATM) and Chk1 or Chk2 are variably capable of inducing a G1/S phase block by inhibiting cdk2, cyclin E, or E2F/Dp. The embryonic fruit fly hypoxia-responsive segment of the cycle (interphase arrest) is delineated by the central arrowhead. G0, quiescent or differentiated state; G1, gap phase 1; S phase, DNA synthesis; G2, gap phase 2. Components of mitosis are as follows: P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase; CK, cytokinesis.](image-url)
A major step in the understanding of cell cycle progression and regulation was the purification of MPF or mitosis promoting factor (also known as the M phase-specific promoter and meiosis promoting factor). The kinase activity of MPF was shown to oscillate in phase with the major transition points of the cell cycle and be absolutely required for transit through the cycle (118). Even more significant progress in understanding cell cycle progression was made when the composition of MPF was determined to be the complexing of a protein kinase cdk1, also known as histone 1 kinase or cell division cycle (cdc) 2, to an activating, regulatory subunit, cyclin A. Another major breakthrough occurred when it was determined that the catalytic or enzymatic element (cdk1) of MPF was the cdc2 gene of *Schizosaccharomyces* (S.) *pombe* and cdc28 gene in *Saccharomyces* (S.) *cerevisiae* and is required for the G1/S transition (for reviews, see Refs. 60 and 66). Human cdk1 was later discovered and found to possess a G2/M role. There is apparently a single cdk (cdc2, cdc28) for the major transitions in yeast cells, whereas the G2/S transition in higher eukaryotes is controlled by different cdk homologs (58). In mammals, different cyclins or cdkss assemble at different points in the cell cycle (reviewed in Refs. 92, 129, 161).

Originally, it was assumed that cyclin A was also in a complex with cdk1 (cyclin A/cdk1) and that this complex appeared to oscillate with the same cell cycle kinetics as MPF (cyclin B/cdk1) (46). It was also noted that both cyclin A and cyclin B were degraded at the completion of metaphase. In addition, cyclin B degradation is required for the exit from mitosis. It was therefore determined that the protein kinase cdk1 operated at essentially two major transition points in the cell cycle, G2/S and G2/M, and that phosphorylation and dephosphorylation events were integral components of cell cycle progression. A simple model of the cell cycle was thus generated in which the complexing of cyclin A to cdk1 initiated S phase entry and cyclin B and cdk1 was required for the initiation of mitosis (Figs. 1 and 2).

Unraveling the mysteries associated with progression through the cell cycle was hampered by the apparently promiscuous nature of these proteins (e.g., cdk1 and its multiple partners, cyclins A and B), making it difficult to specify the roles that each played in the cell cycle. However, three characteristics of the cell cycle eventually led to the clarification of the roles of these proteins in cell cycle regulation: 1) sequential cell cycle events required the completion of a prior step to be initiated, 2) new protein synthesis was required to overcome meiotic arrest points in oocytes and to initiate any new mitotic cycle, and 3) protein degradation was an essential component of progress through the cycle (100).

### Regulation of The Cell Cycle: Modern View

Remarkable progress in understanding the molecular mechanisms driving the cell cycle engine has been made since the discovery of the nature of the MPF. With the advent of cloning techniques, mutational analyses, and PCR, our understanding of the cellular mechanisms controlling cell cycle activity, entrance into the cycle, exit from the cycle, and progress through various stages of the cycle have become readily available. *Drosophila* and yeast have played a tremendous role in elucidating these pathways (134). The number of proteins that are known to participate in the regulation and execution of the cell cycle has grown exponentially during the past two decades. Below, we will present a simplified overview of what is currently known about cell cycle regulatory proteins and the stages of the cell cycle that they impinge on. It should be noted that many outstanding, in-depth re-
views have been written that report on each stage of the cell cycle.

Cells in G0 can be stimulated to return to the cell cycle and thereby reenter G1 (146). Factors such as mitogens can trigger cyclins D and E to initiate the transcription of genes and the accumulation of protein products necessary to initiate DNA synthesis and chromosome duplication and traverse the G1/S boundary. Clearly, there are several stages of the cell cycle involved, and below we describe them as well as some of the key elements that regulate them.

G1/S transition. During G1, several transcriptional and translational events are coordinated so as to initiate DNA replication. Cells are normally induced to leave G1 and enter S phase by mitogenic agents such as growth factors. These extracellular signals are transduced by "membrane receptors" that activate the translation of the D-type cyclins (cyclins D1, D2, D3 in mammals) and their cognate protein kinase partners, cdk4 and cdk6 as well as cyclin E and cdk2 (Fig. 1).

The first cyclin or cdk holoenzymes activated during the transition from a quiescent state, i.e., G1 or G0, to an actively dividing state are the D-type cyclins and cdk4 and cdk6 (125, 127). This expression is stimulated by growth factors and can be blocked by anti-cyclin D antibodies to yield a pre-S phase block, indicating that cyclin D is required from middle to late G1 (6, 152). There is some lack of clarity as to whether cyclin E and/or cdk2 is generated simultaneously with or soon after cyclin D and/or cdk4,6. The expression of cyclin D and E is controlled by a triad of proteins: the E2F1 (elongation factor of E1A virus, E2F1–6 in mammals), Dp1 (dimerization partner, Dp1, Dp2), and the retinoblastoma protein product (RBF or pRb) (reviewed in Refs. 50, 177). During a majority of the cell cycle, RBF is hypophosphorylated and is conjugated to the E2F1/Dp1 dimer and functions to inhibit the S-phase-inducing transcriptional activity of E2F1/Dp. In late G1, RBF is hyperphosphorylated, possibly by cdk2, and removed from the E2F1/Dp1 dimer, allowing E2F1/Dp1 to initiate transcription of genes required for S-phase entry, such as cyclins D and E (110, 135) (Fig. 1). Cyclin E is presumed to be synthesized after cyclin D and demonstrates peak protein expression in late G1. Cyclin E associates with cdk2 (102) and functions to initiate the transcription of S-phase genes and their products. Cyclin E kinase activity also peaks in late G1, Cyclin E levels oscillate periodically during the cell cycle from G1 to S phases and return to low levels in G2 (125, 127).
cycle, and cyclin E is degraded when cells enter S phase. The microinjection of anti-cyclin E or cdk2 antibodies leads to a G1 arrest (85, 140, 171).

Cyclin D and cyclin E are somewhat redundant in that they complement G1 cyclin yeast mutants and both have a role in G1. Ectopic expression of both cyclins D and E accelerates G1 phase and are therefore rate limiting (reviewed in Refs. 85 and 161). Cdk2 can complement or rescue yeast cdk1 mutants (53, 126, 136). On the other hand, cdk4 and cdk6 cannot complement cdk1 mutants and therefore are considered to be more highly evolved. Utilizing dominant-negative mutants, it was demonstrated that cdk1 mutants arrest at G2/M, cdk2 and cdk3 mutants arrest at G1, and cdk4 and 6 mutants do not arrest (172).

S phase. The retinoblastoma protein product (RBF or pRb) is the most well studied of the cell cycle regulatory proteins. It was first noted to be absent or inactivated in eye tumors (retinoblastomas) and is also found in small cell lung cancer, sarcomas, and bladder cancer (91). RBF is targeted by mammalian viral tumor proteins such as E1A from adenovirus, T antigen from simian virus 40, and E7 from human papilloma virus, which binds directly to inactive, hyperphosphorylated RBF (49, 89, 128) and therefore enhances E2F activity which binds directly to inactive, hyperphosphorylated RBF (49, 89, 128) and therefore enhances E2F activity leading to an accelerated G1/S. The hypophosphorylated form of RBF is the active form found bound to E2F1/Dp in quiescent cells and in early G1 and thereby exerts its repressive function. During mid- to late-phase G1, RBF is hyperphosphorylated and removed from the E2F1/Dp dimer, therefore inactive and permitting the initiation of S phase by E2F1/Dp. Later in the cycle, Cyclin D targets RBF to be dephosphorylated by the phosphoprotein phosphatase type 1 so that it can once again repress E2F1/Dp activity through complexation (121). Therefore, cells without RBF or with compromised RBF function do not have cyclin D regulation and demonstrate increased oncogenic potential.

On release from RBF, E2F1, in conjunction with its dimerization partner Dp, activates S-phase genes (reviewed in Refs. 110 and 135) when stimulated by cyclins D and E (45, 57). E2F proteins can also be complexed with other RBF-related proteins such as the pocket proteins p107 and p130, which can also modify the transcriptional activity of E2Fs (56, 78, 112). p107 and p130 can also act as substrates of cyclin D, and both p107 and p130 associate with E2F4 (8, 63). The emerging model of E2F activity is that different E2Fs are activated sequentially in G0/G1 and G1 (8, 29, 63). For example, p107 is an E2F regulator that substitutes for p130 in late G1. Cyclin A/cdk2 then turns off E2F/Dp (48, 105). Overexpression of either p107, p130, or RFB leads to a G1 arrest, whereas mutations in E2F and Dp lead to delay or arrest, respectively.

The transcription of S phase genes leads to chromosomal duplication. On accumulation of required protein products such as the origin recognition complex (ORC) and DNA polymerase, cells undergo chromosomal duplication (41, 167). DNA replication involves the precise duplication of chromosomes during S phase of the cell cycle, which is accomplished by replication forks generated at ORCs, probably at single sites in lower organisms and at thousands of sites in the eukaryotic genome (21, 47). There are a number of S-phase proteins involved in this process. The ORC binds to chromatin at potential sites of initiation (43). Subsequently, the replication licensing factor prepares the initiation site, and the SPF, a cdk (cdc6 in S. cerevisiae and cdc18 in S. pombe) that functions at the G1/S transition, then triggers the initiation of DNA replication at the initiation forks. To ensure chromatin material is only duplicated once during each cycle, other cdk5 prevent reinitiation of DNA replication.

Progress through S phase. Cyclin A, whose kinase activity was detailed in S. pombe, is synthesized in late G1. Although cyclin A is synthesized late in G1, its kinase activity can be detected in the S phase. The microinjection of anti-cyclin A antibodies or anti-sense oligonucleotides directed against cyclin A inhibits DNA synthesis, indicating that cyclin A is required for DNA synthesis (39, 108). The cyclin A/cdk2 complex localizes to nuclear/chromosomal replication foci (16). However, cyclin A also demonstrates peak kinase activity in G2 and is required for entry into mitosis. Cyclin A binds cdk2, whose peak kinase activity occurs in G2. Cyclin A is suddenly degraded thereafter. Therefore, cyclin A/cdk2 activity is required both at the G1/S and G2/M transition points (143).

Having completed the S phase, cells then enter another gap phase, G2, before the initiation of mitosis. During this period, MPF is generated and accumulated. The activity of this holoenzyme, via its ability to activate several other proteins, many of them kinases, permits NEBD, spindle formation, and chromatid segregation.

G2/M transition. Originally, it was thought that entry into mitosis is triggered solely by MPF, which is composed of cdk1 and cyclin B. However, soon it was appreciated that entry into, progress through, and exit from mitosis are catalyzed by the accumulation and activation of both cyclin B/cdk1 and cyclin A/cdk2. Cyclin B/cdk1 is absolutely required for mitotic entry, whereas cyclin A/cdk2 appears to be necessary for the entry into prophase as well as progress through mitosis. Under the dually opposing control of cdc25, a phosphatase that activates cdk1, and wee1, a kinase that inhibits cdk1, initiation of mitosis is achieved by accumulating cyclins A and B while inhibiting wee1 kinase and dis inhibiting cdc25 (15) (Fig. 2).

Mitosis. Prophase. Furuno et al. (61) suggested that cyclin A/cdk2 is able to move cells through most of prophase but that cyclin B/cdk1 activity is required at the end of prophase for NEBD. Cyclin A/cdk2 is therefore necessary for the initiation of chromosome condensation, disassembly of nucleoli, and the activation and perhaps translocation of cyclin B/cdk1 into the nucleus. On activation and translocation of cyclin B/cdk1 to the nucleus, cells enter metaphase, which involves the completion of chromosome condensation, NEBD, and spindle formation.

Metaphase. During metaphase, the sister chromatids are aligned along the equatorial plate of the cell. On
attachment of all the kinetochores, the tension generated by the activity of several kinesin motor proteins and dyneins allows the chromatids to be pulled to opposite sides of the cell. Sister chromatids are held together at the equatorial plate by several protein complexes, including cohesins and securins (reviewed in Refs. 32, 181, and 182).

METAPHASE-TO-ANAPHASE TRANSITION. The metaphase-to-anaphase transition requires the activation and inactivation of several cell cycle regulatory proteins (31, 100, 133, 182). The multisubunit ubiquitin ligase, known as the cyclosome or anaphase-promoting complex (APC/C), ubiquinates the regulators of sister chromatid cohesion such as pds1 in budding yeast and cut1 in fission yeast, which are then degraded by the p26 proteasome (25). Sister chromatid separation is controlled by the degradation of sister chromatid cohesion factors. APC/C-dependent proteolysis of the sister chromatid cohesion complex, especially that of the yeast anaphase inhibitor pds1 (32), is currently believed to be mediated by the WD40-repeat protein cdc20 (Fizzy in D. melanogaster and Slp1 in fission yeast). The APC/C also participates in the exit from mitosis by degrading the mitotic cyclins A and B, sequentially. It is postulated that cyclin A/cdk2 is responsible for the activation of the APC/C, thereby ensuring its own destruction and subsequently that of cyclin B. However, the release from metaphase does not appear to require the ablation of cyclin A activity; rather, the release from metaphase requires the degradation of cyclin B and the inactivation of cdk1 by ubiquitin-dependent proteolytic activity of the APC/C (101, 130, 168). Another WD40-repeat protein, Cdh1/Hct1 (Fizzy-related in D. melanogaster) is more important for the degradation of mitotic cyclins and other APC/C substrates beyond anaphase and during G1 (23, 24).

TELOPHASE/CYTOKINESIS. After completion of this maneuver, the cell undergoes cytokinesis, whereby a variety of cytoskeletal proteins, namely actin, pinches the cell in half to generate two daughter cells with equivalent quantities of DNA and generally equal amounts of cytosolic material (64, 76). Yeast are the exception in that budding results in unequal segregation of cytosolic elements.

Cdk inhibitors. Another level of cell cycle regulatory control involves inhibitors of cdk activity, the cdk inhibitors (CDKIs). These include members of the CIP/KIP family (p21, p27, p57), which are presumed to inhibit all G1-phase cyclin/cdk complexes, and members of the INK4 family (p16, p15, p18, p19), which are thought to inhibit only cyclin D/cdk complexes (reviewed in Ref. 183). On activation by p53 during a variety of stresses, p21 (Cip1, Waf1, or Sdi1) is able to bind to and directly inhibit a number of cyclin/cdk complexes including cyclin E/cdk2 and cyclin A/cdk2 but not cyclin B/cdk1 (Figs. 1 and 2) (80). p21 also prevents S phase and G2 cells from entering mitosis and causes early prophase cells to return to interphase (G2) (61). Mutations in the CDKIs have been implicated in several tumors (98).

Checkpoints

Checkpoints are agents that function as growth suppressor genes, and the loss or mutation of these agents leads to cancer (84, 174).

The DNA replication and damage checkpoints. Cell division is controlled by checkpoint controls that ensure that DNA is completely replicated (DNA replication checkpoint) and undamaged (DNA damage checkpoint) before cell division occurs (9, 54). The DNA damage checkpoint activates pathways that delay cell cycle progression to allow for the repair of damaged DNA (82). The G1 DNA damage checkpoint arrests cells in G1 before DNA synthesis, whereas the G2 DNA damage checkpoint arrests cells in G2 before mitosis (Figs. 1 and 2) (185). Activation of the DNA damage checkpoint involves the phosphoinositide kinase homologs ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-related protein (ATR), which functions at both G1 and G2. The DNA damage checkpoint imposes a block at the G1/S transition point by inducing ATM, ATR, chk1 and 2 protein kinases, and p53 (Fig. 1), which subsequently activates p21 (51). Integral to the G1 arrest is the p53 transcription factor (13, 99) (Figs. 1 and 3). During exposure to IR and UV, p53 is stabilized by ATM and activates many genes and the CDK1 p21 (12, 52, 173), which blocks transcription of cdk complexes required for S-phase entry. There are, however, p53-independent mechanisms that are also required for G1 arrest in response to DNA damage (1). It is interesting to note that recent studies have linked p53 and hypoxia-inducible factor 1 (HIF1) activity, indicating that p53 is able to directly bind to HIF1α and destabilize its expression by promoting ubiquitination mediated by mouse double minute chromosome Mdm2, which suppresses p53, and proteosomal degradation during hypoxia (139, 154). Conversely, during hypoxia, HIF1 can produce apoptosis via p53-dependent pathways and can provoke a G1 arrest by upregulation of CDKIs (p21, p27) and subsequent hypophosphorylation of RBF (37, 62, 106, 163) (Fig. 3). The interaction of HIF1 with several signaling pathways has recently been discovered and will be discussed below.

In contrast to the G1 arrest, G2 arrest incurred by DNA damage does not require p53. In the absence of p53, gene transcription does not contribute to a G2 arrest after DNA damage (33). Instead, the DNA damage checkpoint delays the accumulation of mitotic proteins and thereby induces a G2 arrest (33). The G2 checkpoint appears to act by preventing the cdc25c (mammalian, String in D. melanogaster) protein phosphatase from activating the cdk1 protein kinase (147, 184). This occurs by maintenance of cdc25 in a phosphorylated form which is bound by 14-3-3 protein and thereby prevents cdc25 from accumulating in the nucleus and activating cdk1 (35, 68, 107, 119). Ultimately, this results in maintaining cyclin B/cdk1 in a phosphorylated and inactive state and imposes a block at the G0/M transition (Fig. 2). However, the extended maintenance of a G2 arrest does require p53 (18).
The spindle checkpoint. The spindle checkpoint is activated to inhibit cell cycle progression when mitotic spindles are disrupted or chromosomes have not attached to microtubules. The mechanism of inhibition involves the binding of components of the spindle checkpoint pathway (e.g., Mad and Bub proteins) to the ubiquitin-conjugating system and subsequent inhibition of the APC/C, which is normally involved in sister chromatid separation (Fig. 2).

The spindle checkpoint is responsible for ensuring the accurate separation of sister chromatids. Genetic screens in budding yeast (S. cerevisiae) have identified several members of the spindle checkpoint, including Mad1, 2, and 3; Bub1, 2 and 3, Mps1; cdc55; and cdc20 (Fizzy in D. melanogaster) (4, 38). Mutations of these genes lead to sister chromatid separation in the presence of defective mitotic spindles or unattached kinetochores, which can lead to aneuploidy, cell death, or cancer.

The spindle checkpoint is activated by spindle abnormalities to prevent the metaphase-to-anaphase transition. Studies in S. cerevisiae, S. pombe, Xenopus, and mammalian cell lines have revealed that the spindle checkpoint blocks the activation of the APC/C in response to a single unattached kinetochore and in response to a lack of tension between the sister chromatid and spindle pole or by extensive spindle damage induced by microtubule depolymerizing drugs such as nocodazole (113, 114).

Two protein kinases, Mps1 and Bub1, are required for cell cycle arrest in response to spindle defects and serve to activate Mad1 via phosphorylation. The Mad-Bub complex is recruited to unattached kinetochores and inhibits the activation of the cdc20-APC/C complex necessary for sister chromatid separation. This results in a metaphase arrest. Cdc20/slp1 physically interacts with components of the spindle checkpoint pathway such as Mad1, 2, and 3, and the interaction between Mad2 and cdc20/slp1-APC/C inhibits the ubiquitination activity. The current model of the spindle checkpoint is depicted in Fig. 2.

The prophase checkpoint. A newly uncovered checkpoint, the prophase checkpoint, has been recently described. It is noteworthy that this required in vivo imaging to detect; however, this phenomenon has been reported previously in 1969 (17). Yet, this underscores the importance of the utility of the new technological advances that have been made in the recent past that reveal phenomena not previously amenable to investigation. A key facet of the prophase checkpoint is that cells that have initiated mitosis but have not progressed beyond prophase return to a premitotic stage, i.e., interphase, in response to chromosomal damage (61). Cyclin A/cdk2 appears to be the mediator of the prophase checkpoint, in line with its ability to be inhibited by p21. Reider and Cole (155) were also able to demonstrate that PtK1 cells in prophase returned to interphase if damage was induced <30 min before NEBD.

**THE DIRECT EFFECT OF HYPOXIA ON CELL CYCLE ACTIVITY**

**Animal Models**

Presently, the study of the influence of hypoxia on cell cycle activity has entered a new age. The ability to perform in vivo studies of cell cycle activity via improved imaging capabilities and the use of green fluorescent protein (GFP) technology has revolutionized the study of the biology of the cell cycle. Living organisms, such as embryos, can now be subjected to various paradigms (including hypoxia), and their growth can be observed in real time.
Over the past few decades, it has become apparent that several species, such as the turtle and brine shrimp, are capable of surviving extended periods of severe hypoxia or even anoxia (26, 88, 93). Several years ago, we discovered in our laboratory that *D. melanogaster* is also capable of surviving and recovering from several hours of prolonged and total O₂ deprivation (74, 123). Our discovery of the *Drosophila* O₂ tolerance is important for several reasons: 1) *Drosophila* is genetically very well studied and a large number of different fly lines and mutants are available for dissecting different signaling pathways; 2) it is now becoming clear that in almost all aspects of biology, from ion channels (94) to embryonic development and to neuronal development (5, 75), mammals and *Drosophila* use very similar complements of genes; and 3) with the completion of the sequencing of the entire *Drosophila* genome and now a significant portion of the human genome, the *Drosophila* becomes an increasingly useful model for deciphering the function of certain genetic pathways. It is therefore possible that by understanding the molecular mechanisms underlying the tolerance to anoxia in *Drosophila*, we will be able to better understand mechanisms of intolerance and injury in other species. More importantly, because studies have shown that tumor cells in humans can be very tolerant to anoxia (34), these studies raise the distinct possibility that cell manipulation can induce anoxia tolerance in human cells. This has recently panned out in some of our studies (Chen and Haddad, unpublished observations). Utilization of fruit flies as a model has already proven to be very useful in our studies in both adult flies (74, 122, 123) and embryos, in which we have addressed a number of very interesting and important questions.

Hypoxia induces variegated effects on early rapidly cycling embryos of different species and can affect the cell cycle in a variety of ways. The cell cycle can halt completely and irreversibly, leading to cell death. On the other hand, the cell cycle can halt its activity for a period of time during stress and can resume its activity when that stress is removed. Recovery from such a cessation of activity can be without a price or can compromise cell function in either the long term or the short term. There are many examples of cells that can transiently arrest cell cycle activity. Immortalized cells of human tumors and other mammalian cell lines have been demonstrated to arrest or delay cell cycle during a variety of stresses. For example, exposure to UV, IR, and spindle-disrupting drugs like nocodazole can lead to arrest (138). The length of the arrest and the sequelae that are injurious in the short and long term appear to depend on a multitude of factors, including the age of the organism, the species, the stage of development and the preexisting cell cycle status of the cell. In addition, the duration of the stress as well as the intensity of that stress also play important roles in the nature of the arrest and the extent of injury experienced. Within the cell cycle of both prokaryotes and eukaryotes are a number of transition states that can be interrupted if certain requirements have not been met or if the regulation of a number of cell cycle regulatory proteins has been altered (54, 82, 131, 156). *Brine shrimp*, *zebra fish*, and nematodes. There appears to be specific stages within the cell cycle when a potentially survivable arrest can occur. Again, there is species variability, age, and stage dependence in this response. In studies of rapidly cycling embryos, Padilla et al. (142) recently reported that the nematode *Caenorhabditis elegans* can transiently enter a state of “suspended animation” or arrest during all stages of the cell cycle. *C. elegans* blastomeres arrest in interphase, prophase, metaphase, and telophase but not during anaphase. Zebrafish embryos (*Danio rerio*) also enter a state of reversible arrest (141); however, these embryos arrest during S phase and G₂ but not during mitosis. This indicates that there are species-specific hypoxia-induced checkpoints. Although the stages of cell cycle arrest were not addressed in these studies, it is remarkable that brine shrimp embryos (*Artemia franciscana*) can enter a quiescent state for several years and resume development on reoxygenation (27, 28, 77).

**Fruit flies.** In our particular paradigm, utilizing in vivo analysis of fruit fly embryos containing a GFP-kinesin construct that permitted real-time visualization of the response of fruit fly embryos to hypoxic and anoxic exposure, it was interesting to note that embryos arrested in only two positions in the cycle, i.e., at metaphase (with aligned, nonsegregated chromatids) and prior to the S phase (in G₁ or an hypoxia-induced interphase), as early cycling embryos do not possess a G₁ phase (42, 44) (Fig. 4). These arrest points depend on the stage of the cell cycle when the embryo was exposed to O₂ deprivation; i.e., interphase arrest occurs if hypoxia is induced after metaphase accompanied by some progression through the cell cycle and metaphase arrest is induced if hypoxia is applied shortly before metaphase and can be extremely rapid.

At other stages of development and age, however, there appears to be a more general arrest that occurs in all stages of the cell cycle in the fruit fly. For example, Foe and Alberts (59) reported that *Drosophila* embryos arrest at most stages of the cell cycle, which differs from the data that we have collected. The potential reasons for this discrepancy may involve the nature of the studies, i.e., in vivo vs. in vitro, as well as the level of hypoxia and anoxia achieved during experimental manipulations. Recently, our laboratory reported (44a) that hypoxia of 5% O₂ concentration or greater has no effect on cell cycle activity in *D. melanogaster* embryos; however, at a level of 2% O₂, hypoxia leads to an extreme prolongation of the cell cycle without leading to a cessation of cell cycle activity. Again, it is noteworthy that the use of GFP technology has probably shed light on a phenomenon that has not yet or heretofore been observed. This underscores the relevance and importance of in vivo observation of biological events in real time to truly assess their function. Near anoxia or actual anoxia appears to be required to actually induce cell cycle arrest in our embryos. It appears that there are particular points in the cell cycle, i.e., metaphase and pre-S phase, that are uniquely sensitive to O₂.
deprivation. Therefore, we and others have identified potential cell cycle checkpoints that are responsive to O2 levels in Drosophila embryos. We refer to these as hypoxia-sensitive checkpoints.

In other species, sites of cycle arrest appear to differ. It would seem that each species may have unique points in the cell cycle when a transient arrest can occur, whether limited or global. Recent studies in the C. elegans have supported this notion, as this species is rather sensitive to hypoxia in almost every stage (142).

A number of DNA replication checkpoint proteins have been implicated, and some could be important in transducing the hypoxia signal. These include cdk1 (cdc2), 2, 4, and 6; cyclins A, D, and E, the retinoblastoma protein, and CDKIs (66, 108, 110). On the other hand, components of the spindle assembly checkpoint such as pds1, Mad1 and Mad2, and Bub1, 2, and 3 could play significant roles in an hypoxia-induced arrest (133).

Role of Hypoxia in Tumor Cell Cycle Activity

The role of hypoxia in the establishment or induction of neoplastic transformation, the progress of tumor growth, and the metastatic potential of a solid tumor has recently been extensively investigated (for review, see Refs. 81, 175). Additionally, the role of cell cycle regulatory proteins in the induction of tumors has been studied for some time; however, there has been a recent increase in the appreciation of the variety of cell cycle proteins that contribute to oncogenesis (reviewed in Refs. 83, 92, 111, 162). However, understanding the resistance demonstrated by many solid tumors to various chemo- and radiotherapeutic strategies and the implication of hypoxia and its sequelae in the protection of the tumor and propagation of the tumor cell potential will be the focus of this section.

The first studies of the direct effect of hypoxia on cell cycle activity were performed generally on transformed or oncogenic cell lines. The resistance of hypoxic cells in solid tumors to radiotherapy and chemotherapy had already been well documented (14, 170). It was noted that hypoxic cells in experimental tumors of varied origins could comprise from a few percent up to >80% of the viable tumor cell population (71). After irradiation, some tumors were able to reoxygenate, but this reoxygenation is variable in extent and time course (97). This led to the study of the effect of hypoxia on cell cycle activity and progression and cell viability in a variety of mammalian and human cell lines.
Shrieve and Begg (165) reported that rapidly induced, extreme hypoxia was able to immediately arrest V79–379A fibroblast cells in all phases of the cell cycle, similar to reports of Shrieve et al. (164) in EMT6/SF cells and by Petterson and Lindmo (149) in the human cell line NHIK3025. Utilizing bromodeoxyuridine (BrdU) incorporation and flow cytometry, Shrieve and Begg (165) were able to demonstrate that cells did not move into or out of S phase during hypoxia. Reoxygenation led to the initiation or completion of DNA synthesis; however, subsequent cell cycle progression was markedly delayed. Shrieve and Begg also reported that there appeared to be a slight progression of S-phase cells toward G2. It was also noted that some early S-phase cells did continue to synthesize DNA during the first 2–4 h of hypoxia. During reaeration, cell cycle kinetics were apparently disturbed such that cell cycle progression was markedly slowed and some S phase cells demonstrated endocycling or overduplication of DNA. Similar reports were provided by Åmellem and Pettersen (3).

However, other reports indicated that cells have the capacity for continued growth in the nominal “absence of oxygen” (7, 11, 36, 158). Shrieve and Begg (165) believed that those differences were due to continuous O2 contamination in the other studies. They concluded that a lesser degree of hypoxia or a more gradual induction of hypoxia could lead to different cell cycle activities and arrest points. Another observation of significance was that cell cycle perturbations that occurred during reoxygenation such as cell cycle slowing and endoreplication were dependent on the phase of the cell cycle that cells were in during hypoxia, in agreement with the studies of Petterson and Lindmo (149) and Shrieve et al. (164). The value of these previously mentioned studies was the revelation that cells can delay or arrest cell cycle activity during near anoxia and can continue cycling on reoxygenation. Furthermore, the stage of the cell cycle at the time of hypoxia induction may influence the stage at which the cell arrests.

Several questions remain to be answered, and new questions were generated by these early studies in cancerous cell lines. For example, why and how did cells survive near anoxia, and why did these cells not undergo apoptosis? Some of the answers had to wait for the molecular biology revolution that occurred in the 1980s and 1990s to be answered. For instance, it was realized that several cancers and many tumors cells did not have a functional p53 protein or RBF protein and were therefore unable to induce p53-mediated apoptosis or a RBF-induced G1 arrest (99, 106). This led to a revolution in the understanding of regulation of the cell cycle machinery during normal development and dysplasia and neoplasia.

**Hypoxia, Arrest of Cell Cycle, and Various Mechanisms**

What are the potential mechanisms involved in arrest, cell proliferation, and division during hypoxia? A number of ideas have been generated about potential mechanisms that can play a role in inducing arrest. It is important at the outset to realize that possibly multiple mechanisms could operate in multiple conditions and that different cell types may have varied ways of inducing cell arrest. Of additional importance is that various cell types or cell lines in vitro can halt or delay their cell cycle or inhibit their growth at various levels of O2 and possibly some cells are more sensitive to low O2 than others in terms of the cell cycle. In vivo studies in tumors have shown that cells that are hypoxic are not dividing; other potential stresses in vivo may play a role, but the idea at present is that hypoxia, at least in part, affects cell growth in vivo in tumors (Fig. 3).

There have been a number of potential mechanisms of arrest during stressor hypoxia, with some identified in mammalian cell lines and some in invertebrates. First, O’Farrell and colleagues (42) recently showed that ATP levels and nitric oxide play a role in syncytial embryos, which not only arrest but actually die when they are subjected to hypoxia, in contrast to cellularized embryos that arrest and survive but in which nitric oxide and ATP are not major players. Second, studies have shown that ascites tumor cell line exposed to hypoxia do not progress through the G1 phase and do not synthesize DNA because of depletion of deoxynucleotides from an inhibition of enzymes, such as ribonucleotide reductase and dihydroorotate dehydrogenase, responsible for the synthesis of pyrimidines and DNA bases (10, 116, 117). Most likely, this mechanism cannot play a role in organisms such as D. melanogaster embryos, which arrest within seconds of instituting hypoxia, since there are usually pools of purines and pyrimidines from which DNA can be synthesized. Third, other studies have demonstrated that the initiation or replication of DNA may be sensitive to hypoxia and that this is the reason for the cell cycle arrest (151). Fourth, more specific mechanisms include, for example, the effect of hypoxia on RBF phosphorylation and whether the hypophosphorylation state of RBF found in hypoxia (106, 120) could be a mechanism by which the arrest during G1 takes place. The effect of hypoxia on the rest of the important proteins that control the G1/S transition, which include E2F, Dp and cyclins D and E, is ill defined and not well studied. Furthermore, DNA damage checkpoint proteins may play an important role in the G1/S arrest. If there is any “damage” to DNA during hypoxia or ischemia (which in some cell lines may take place not over minutes but hours), cells may activate mechanisms that would protect them from defects in heritability. Hence, sensor proteins like poly-ADP-ribose polymerase, Rad1, Rad9, Hus1, or Rad17 and their homologs may be important for hypoxia studies. Similarly, kinases such as ATM and ATR, proteins such as Chk1 and Chk2, and downstream effectors (cdc25 and cdk5) may be crucial in the study of the overall sensing and transduction of hypoxia into an arrest (Figs. 1 and 2).

Other potential mechanisms for cell cycle arrest have been studied in our laboratory. Our laboratory has shown (44) that the cell cycle can arrest in hypoxia.
not only in the pre-S interphase but also in metaphase; therefore, proteins such as the securins and separins, which control sister chromatid cohesion and separation as well as a block on the APC, could be involved and perhaps critical in this hypoxia-induced metaphase arrest (90, 104). Our laboratory (44) has also demonstrated that cyclin B is not degraded during a hypoxia-induced metaphase arrest; therefore, it can be hypothesized that O2 deprivation imposes a block on cyclin B degradation either by maintaining the inactivated state of the APC or by preventing activation of the APC.

Metabolic theories also exist. Although these do not pinpoint the exact location of the arrest in the cell cycle, cell division is involved. For example, fructose 2,6-bisphosphatase when overexpressed decreases glycolysis and delays the progression of the cell cycle. It is interesting that this effect of the fructose 2,6-bisphosphatase was mostly related to its kinase domain that was being upregulated alone in cells in vitro (148). Other studies have investigated the effect of hypoxia on insulin-like growth factor (IGF) binding protein and how this may affect cell growth and division in the fetus in utero or in cells in vitro (Fig. 3) (150, 169). Also, recent studies showed that hypoxia inactivates glycogen synthase kinase via phosphoinositol 3 (PI3)-Akt pathway (20), which can impact cell division and proliferation. For instance, previous studies suggested that the activation of this Akt pathway modulates cell cycle arrest. p21 and p27, for example, do not play a major role in the cell cycle arrest but in the cell cycle reentry. Indeed, cells lacking p21 resume DNA synthesis more rapidly with reoxygenation than p21+/− cells. p27 may have also a similar role in applying "brakes" in cell cycle reentry.

Although many studies have been performed to investigate the hypoxia-induced cell cycle arrest, only a few studies have determined some of the pathways responsible for reentry into the cell cycle after such an arrest. It seems from these studies that such mechanisms are not similar necessarily to those that induce cell cycle arrest. p21 and p27, for example, do not play a major role in the cell cycle arrest but in the cell cycle reentry. Indeed, cells lacking p21 resume DNA synthesis more rapidly with reoxygenation than p21+/− cells. p27 may have also a similar role in applying "brakes" in cell cycle reentry.

One of the exciting and interesting questions in this area is the effect of hypoxia on development in very early life. Although many investigators have posed similar questions in the past, we have started to appreciate how important is the titration of O2 in early life for proper development of organs and body structure. Despite the fact that fetuses are naturally hypoxic, further hypoxia or oxygenating fetuses above their usual level of O2 can induce developmental defects (19). How development takes place at high altitude, what are the effects of various altitudes, and what are the functional and structural consequences at high altitude are not well studied at present. Some of the corollary issues in this regard is the effect of hypoxia on cell size vs. cell growth. It is clear now, as has been commented on above, that a number of genes are important in the control of cell size and cell cycle timing and growth. For example, overexpression of E2F or RBF does not increase the size of the organ but modulates the number of cells and therefore cell size in that particular organ (134). The insulin-IGF-P13 kinase pathway in both vertebrates and flies plays a major role in the control of cell size (30). How that pathway is involved in the hypoxia-induced growth changes that are both clinically and experimentally observed is not well studied at this stage. In addition, it is not clear whether the nutrient-activated, rapamycin-sensitive protein kinase pathway is involved during hypoxia and how that affects cell growth and

nuclear transcription, which in turn limits adipogenesis. HIF−/− cells do not respond to hypoxia-induced inhibition because a downstream gene to HIF, DEC1/Stradi, does not get activated by HIF and does not repress uPAR to inhibit adipogenesis. Hence, hypoxia inhibits adipocyte differentiation. Knowing the mechanisms that can lead to obesity may allow us to control this disease by potentially mimicking the effect of low O2.

It should be clear from the preceding remarks that there are a number of genes and proteins that control the hypoxia-induced changes in cell and tissue phenotype. For example, a number of genes determine the influence of hypoxia on cell proliferation or cell cycle arrest and apoptosis. How the mitochondrial-signaling pathway of Apaf/caspase 9 leads to cell death via caspase 3 is involved in signaling of the cell cycle is not clear at present especially when it is related to hypoxia and cytochrome release. There is evidence, however, that this Apaf-pathway is independent of p53 and bax (2).

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size. Other questions regarding development and adaptation at high altitude across multiple generations have not been reported.

Hypoxia, as a stressful stimulus, has taken a different meaning in the past decade. Because of the nature of the research done in hypoxia on so many different types of cells, in vertebrates and invertebrates, and in so many different aspects of cell biology and physiology not only in healthy tissues but also in diseased or mutated cells, we have learned how to take advantage of the effects of hypoxia to treat diseases. For example, the scientific community has taken advantage of what hypoxia does to tumor blood vessels to combat tumors via antiangiogenic drugs. Because we now know that tumors have hypoxic cores, Liu and colleagues (115) genetically engineered anaerobic bacteria that target hypoxic regions of solid tumors. These bacteria carry enzymes that transform into cytotoxic substances when bacteria, the vectors, reach a hypoxic environment. It will be fascinating and certainly important to exploit further our understanding of what genes hypoxia induces to better treat human diseases.

SUMMARY

Hypoxia induces many effects on cells and tissues. In this review, we have briefly outlined some of the salient features of the effect of hypoxia on cell cycle in both vertebrates and invertebrates. We have also highlighted the importance of D. melanogaster as an important model in trying to understand the effects of hypoxia on cell division, growth, and proliferation. Our hope is not only to stimulate interest in this area of investigation but also to show that the understanding of disease at the molecular level for use in new therapeutic modalities presents an exciting future.

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