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草鱼核因子E2相关因子2基因克隆分析及其对呼吸爆发的调控作用

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摘要: 呼吸爆发过程可以产生大量的活性氧, 核因子E2相关因子2(*Nrf*2)在此过程中起着重要作用。实验克隆和分析了草鱼的*Nrf*2基因, 随后制备了Nrf2蛋白的多克隆抗体, 研究了其和呼吸爆发的关系。草鱼*Nrf*2基因cDNA长度为1994 bp, 开放阅读框为1782 bp, 编码593个氨基酸(aa)。氨基酸序列比对发现, 草鱼*Nrf*2基因与鲤的同源性最高, 为87%; 草鱼*Nrf*2基因含有6个进化过程中保守的Neh [Nrf2-Epoxy chloropropane (ECH) homology]区。实时定量PCR (qRT-PCR)和蛋白印迹法(Western blot)检测结果表明, *Nrf*2基因在检测的草鱼8个组织中均有表达。Nrf2激活剂叔丁基对苯二酚(tBHQ)处理草鱼肾细胞(CIK)后, CIK细胞总抗氧化能力显著上调, 产生的活性氧下调, *Nrf*2及其下游的*HO*-1和*GST*基因的mRNA表达上调。Western blot和免疫荧光(IF)检测结果表明, tBHQ处理CIK后, Nrf2的蛋白表达也上调, 并伴随着入细胞核现象。研究表明, 保守的草鱼*Nrf*2基因在机体中广泛表达, 能通过上调自身及其下游抗氧化基因的表达下调细胞活性氧的产生, 从而参与调控呼吸爆发过程。

关键词: 草鱼; 核因子E2相关因子2(*Nrf*2); 基因克隆; 多克隆抗体制备; 呼吸爆发

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核因子E2相关因子2(*Nrf*2)是一种重要的调控氧化应激反应的核转录因子[1], 属于CNC (cap and collar)亮氨酸拉链(CNC-bZIP)蛋白家族[2], 通常和抗氧化原件(ARE)相互作用, 参与调节有氧呼吸、胚胎发育、炎症和癌变等多个生物过程[3-4]。

Nrf2一般含有6个保守Nrf2环氧氯丙烷同源物(Neh)功能域, 即Neh1~Neh6[5]。Neh1通过和多种小分子肌腱纤维瘤(musculo aponeurotic fibrosarcoma, Maf)蛋白结合形成异源二聚体, 从而识别并结合DNA上的抗氧化原件ARE, 启动下游抗氧化基因转录[6]。Kelch样环氧氯丙烷相关蛋白1(Kelch-like ECH-associated protein-1, Keap1)可结合Nrf2的Neh2结构域, 从而抑制Nrf2的转录表达[7]。Neh3、Neh4和Neh5功能域在转录激活过程中也起着重要作用[8-9]。Neh6在细胞应激状态下, 可以维持Nrf2功能的正常运转; 在非应激状态下, 可调节Nrf2的降解[10-11]。正常稳态Nrf2大量存在细胞质中, 少部分在细胞核中转录激活下游基因的表达以维持正常的生理机能[12]。在应激状态下, Nrf2与Keap1分离, 从细胞质迁移到细胞核中调控抗氧化基因和二相代谢酶的表达[13], 如谷胱甘肽S转移酶(glutathione S-transferase,

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GST)和血红素加氧酶-1(hemo oxygenase-1, HO-1)[14]。

呼吸爆发(respiratory burst, RB)，也叫氧爆发，是不同类型细胞快速释放活性氧(ROS)(超氧阴离子和过氧化氢)的过程，在免疫系统中发挥着重要的作用。ROS可以使机体内氧化还原状态发生改变，减少机体抗氧化抵抗力，引起氧化损伤[15]。激活或过表达Nrf2可以减少机体或细胞有害物质的产生，增强抗氧化能力，缓解由ROS引起的疾病[16-19]。Nrf2有许多小分子激活剂，如联苯酚类的叔丁基对苯二酚(tert-Butylhydroquinone, tBHQ)[20]、异硫氰酸酯类的莱菔硫烷(sufloraphane, SFN)[21]、苯醌类吡噻硫酮(oltipraz)[22]。它们能通过激活Nrf2的核转位，特异性上调细胞内防御性物质的含量。

目前，关于哺乳动物的*Nrf*2基因的研究取得了大量的成果[23-25]。在鱼类*Nrf*2的研究上也有一些进展[26]。关于草鱼(*Ctenopharyngodon idella*) *Nrf*2的研究，主要集中在*Nrf*2和营养的关系上。如果饲料中缺乏色氨酸、烟酸、胆碱、泛酸和叶酸等营养物质，会导致*Nrf*2的表达下降[27-31]。草鱼是我国重要的淡水养殖鱼类，年产量超过500万t。草鱼可以被病毒、细菌和寄生虫等多种病原侵染，并导致重大的经济损失。研究草鱼的基础免疫学可以为草鱼疾病的防控提供依据。氧化应激是宿主抵御病原感染的重要机制，而*Nrf*2基因和氧化应激的调控密切相关。因此，开展草鱼*Nrf*2基因和氧化应激的调控研究具有重要意义。本实验根据已报道物种的*Nrf*2保守序列，从草鱼中克隆得到含整个开放阅读框的cDNA序列，制备了多克隆抗体，并对其在不同组织中的表达差异及对呼吸爆发过程的调控作用进行分析，旨在为草鱼Nrf2的深入研究奠定基础。

1 材料与方法

1.1 实验材料

实验用草鱼购自湖北武汉百容有限公司。草鱼暂养于室内半径为0.6 m，深1 m的大圆桶，2周饲养驯化后开展实验。取5尾规格在30~40 g的健康草鱼的脑、鳃、头肾、体肾、心脏、脾脏、肠和肝脏8个组织，分装于RNAiso Plus试剂(TaKaRa公司，大连)和PBS中，冻存于−80 °C备用。草鱼CIK细胞为本实验室保存。叔丁基对苯二酚(tBHQ)购自Sigma公司；总抗氧化能力试剂盒(FRAP法)购自上海碧云天生物技术有限公司；MTT购自Biosharp公司；大肠杆菌菌株DH-5α和BL21(DE3)购自北京全式金生物技术有限公司。

1.2 草鱼*Nrf*2基因的克隆和生物信息学分析

按照RNAiso Plus试剂(TaKaRa公司)说明书提取草鱼各组织总RNA，采用微孔板分光光度计Epoch (Biotek公司，美国)检测RNA的浓度，使用PrimeScript™ RT试剂盒(TaKaRa公司)和Oligo dT引物合成cDNA，−20 °C保存备用。根据NCBI中公布的不同物种*Nrf*2基因序列，设计扩增草鱼*Nrf*2核心片段的简并引物(表1)。PCR反应体系：2×Taq Mix 10 μL，上、下游引物各0.5 μL (10 μmol/L)，cDNA 1 μL，ddH_2O 8 μL。反应程序：95 °C 预变性5 min；95 °C 30 s，58 °C 30 s，72 °C 2 min，35个循环；72 °C 延伸10 min。PCR产物经1%琼脂糖凝胶电泳检测后，用胶回收试剂盒(Aidlab公司，北京)回收目的条带，并连接于pBLUE-T质粒(Vazyme公司，南京)，转化到*E.coli* DH5α中，涂LB(含Amp)平板培养基。翌日挑取单菌落进行菌液PCR检测，将阳性克隆在液体LB培养基培养。挑选阳性菌液送公司测序。将测序结果于NCBI中比对，获得*Nrf*2保守区序列，再以该段序列作为模板进行3′-RACE片段扩增，设计特异性外侧引物和内侧引物(表1)。3′-RACE反转录按照3′-Full RACE core set with PrimeScript™ RTase试剂盒(TaKaRa公司)说明书进行，PCR扩增使用LA Taq试剂盒(TaKaRa，公司)，按照其说明书进行。PCR产物的纯化和序列测定同上述核心片段的克隆。根据其他物种*Nrf*2序列和已有草鱼*Nrf*2序列，设计上下游引物，扩增*Nrf*2基因序列，所用引物见表1。PCR扩增、反应条件、产物的纯化、克隆和序列测定类似于上述核心片段的克隆。

使用下列软件和工具对*Nrf*2基因进行分析：Clone Manager软件对获得的*Nrf*2基因序列进行分析，确定其开放阅读框(ORF)；DNAMAN 6.0软件进行氨基酸序列分析；BLAST工具(https://blast.ncbi.nlm.nih.gov/Blast.cgi)进行核苷酸和氨基酸序列相似性分析；在线软件(http://www.expasy.org/)分析蛋白质的特性；SMART软件(http://smart.embl-heidelberg.de/)预测蛋白质的结构域；MEGA 6.0构建Nrf2系统进化树。

1.3 Nrf2多克隆抗体制备及其特异性检测

Nrf2重组质粒的构建、融合蛋白的表达及检

表 1　实验所用引物及其序列

Tab. 1　Primers used in the experiments

引物名称 primer	序列(5′-3′) sequence from 5′ to 3′	GenBank号 GenBank number	引物信息 information of primers
Nrf2-F1	AGTGAYTCDGACDYGGADGAGATGGA		RT-PCR
Nrf2-R1	AAGTCRTCHACDGGCAGRTTGATGATC		
Nrf2-Inner	CAGTGGCTACTCCAAACCTCCCTTC		3′-Race
Nrf2-Outer	TCAATCTGCCTGTGGATGACTTC		
Nrf2-F2	ACACCCCAGCCTTCACCCAG		RT-PCR
Nrf2-R2	TTCATCTCCTGCTGCTCTGC		
Nrf2-F3	ACACCCCAGCCTTCACCCAG		RT-PCR
Nrf2-R3	TTCATCTCCTGCTGCTCTGC	KX243419	
Nrf2-F4	CMAGYCAACAGGAYATGRA		RT-PCR
Nrf2-R4	GCCGCTGGCCGGAAGGCTT		
Nrf2-F5	TCGGTGAAGGATTACAAA		RT-PCR
Nrf2-R5	CCGCTTCTCCTCCTCCTG		
Nrf2-F6	GATAAATCCCCCGCAGAGCA		qRT-PCR
Nrf2-R6	TCATGTCCACGGTAAACGGG		
β-actin-F	AATTTAGGGAGTGGCCCTGGC	M25013.1	qRT-PCR
β-actin-R	GGAAGTCCCTCTGCATTCTCTC		
HO-1-F	AAAAGGCGTAGACCATCC	KX243425	qRT-PCR
HO-1-R	GTCGCATCACGCAGAAGT		
GST-F	TCTCAAGGAACCCGTCTG	EU107283.1	qRT-PCR
GST-R	AGTCTCAAGGAACCCGTCTG		
Nrf2-XhoI-F	CTCGAGATGAAAGCAAAGAACCCAAA	KX243419	构建重组质粒
Nrf2-EcoRI-R	GAATTCCTAGTTATTCTTAACAAGAG		

测　　根据得到的草鱼*Nrf2*序列，设计引物(表1)扩增保守的BRLZ区域(558 bp)。PCR反应条件：95 °C 预变性5 min；95 °C 30 s，58 °C 30 s，72 °C 45 s，35个循环；72 °C 延伸10 min。将获得的PCR产物按照常规技术连接到pET-32A质粒(TaKaRa公司)，构建含有His-tag的融合蛋白表达质粒pET-32A-BRLZ。将pET-32A-BRLZ转化到*E.coli* BL21(DE3)，使用1 mmol/L IPTG在37 °C条件下诱导8 h，收集菌体后，用PBS按比例稀释，加入5×loading buffer沸水煮沸15 min，上样于10% SDS-PAGE胶，检测融合蛋白的表达。随后用蛋白胶回收试剂盒(TaKaRa公司)和His标签特异性层析柱(Abcam公司，美国)按说明书回收目的蛋白，冻存于−80 °C，备用。将收集到的pET-32A载体菌液和回收纯化的目的蛋白，用PBS按比例稀释，加入5×loading buffer沸水煮沸15 min，上样于10% SDS-PAGE胶，2 h后进行转膜，溶解于TBST溶液的5%脱脂牛奶(BD公司，美国)室温封闭3 h；小鼠抗His-tag单抗(1∶1000比例稀释)室温孵育膜3 h；TBST洗涤5次，每次5 min；再用HRP标记山羊抗小鼠二抗IgG(1∶1000比例稀释)(Cell Signaling公司，美国)孵育膜45 min后，TBST洗涤5次，每次5 min；ECL显色1 min，使用Amersham Imager 600获取照片。

Nrf2多克隆抗体制备及其特异性检测　　将获得的重组蛋白送至中国科学院武汉病毒所免疫大耳白兔，获得Nrf2多克隆抗体。

按每孔2 mL 10^5细胞浓度将CIK细胞接种于

6孔板(Corning, USA)，28 °C、5% CO_2培养箱中培养过夜。PBS洗涤后，用细胞刮收集细胞并离心5 min。100 μL RIPA裂解液(碧云天公司，上海)于冰上裂解细胞沉淀20 min后，按比例加入5×loading buffer沸水煮沸15 min，分装冻存于–80 °C。后续SDS-PAGE胶制备、转膜、抗体孵育、显色等步骤按上述重组蛋白正确性检测过程进行，其中孵育膜的一抗使用自制的Nrf2多克隆抗体，稀释的比例为1∶1000。

1.4 草鱼Nrf2的组织表达分析

草鱼*Nrf2* mRNA的组织表达分析　以获得的*Nrf2*基因序列设计荧光定量PCR上、下游引物，*β-actin*作为内参(表1)。以草鱼脑、鳃、头肾、体肾、心脏、脾脏、肠和肝脏8个组织总RNA为模板，按照PrimeScript™ RT reagent Kit with a gDNA Eraser试剂盒(TaKaRa公司)说明书去除总RNA中的基因组DNA，并反转录合成cDNA，–20 °C保存。实时荧光定量PCR反应使用AceQ qPCR SYBR® Green Master Mix (Vazyme公司)试剂，反应于Roche LightCycler 480(Roche，美国)上进行。其中每个样品的目的基因和内参基因分别进行3次重复。反应条件：95 °C 预变性5 min；95 °C 10 s，58 °C 10 s，72 °C 10 s，45个循环。相对表达数据分析采用$2^{-\Delta\Delta C_T}$法[32]。数据表示方法为平均值±标准差，并采用SPSS 19.0软件中的单因素方差分析(One-Way ANOVA)进行统计分析，使用OriginPro 8.0软件绘制柱状图。

草鱼Nrf2蛋白的组织表达分析　用RIPA裂解液(碧云天公司)按说明书提取草鱼脑、鳃、头肾、体肾、心脏、脾脏、肠和肝脏8个组织的蛋白样，加入5×loading buffer沸水煮15 min，分装保存于–80 °C。按照Nrf2多克隆抗体特异性检测的步骤检验草鱼Nrf2蛋白的组织表达，以β-actin作为内参。

1.5 激活剂tBHQ对CIK细胞存活率、总抗氧化能力和ROS的影响

MTT法检测tBHQ对细胞的毒性　设置tBHQ的浓度为0、1、5、10、20、50、100、200、400、600 μmol/L，每个浓度重复6次。按设置接种200 μL 10^4细胞于96孔板28 °C、5% CO_2培养箱中培养过夜，加入相同体积不同浓度的tBHQ继续培养24 h后，再加入20 μL MTT (5 mg/mL)继续培养4 h。将孔内所有液体吸出，加入150 μL DMSO (Biosharp公司)于摇床中混匀15 min，完全溶解细胞，在多功能酶标仪(Nano Quant公司)波长490 nm处检测其OD值。

FRAP法检测tBHQ处理后CIK细胞总抗氧化能力　接种2 mL 10^5细胞于6孔板28 °C、5% CO_2培养箱中培养过夜，设置对照组和tBHQ (10 μmol/L)处理组，每个处理设置3个重复，时间点为0、3、6、9、12 h。培养至相应时间点，用RIPA裂解液裂解细胞，获得蛋白样。一部分蛋白样用BCA蛋白浓度试剂盒(Fintest公司)检测其蛋白浓度，另一部分用FRAP细胞总抗氧化能力检测试剂盒(碧云天公司)按说明书检测其总抗氧化能力。细胞总抗氧化能力的最终表示方式为每克蛋白重量中的总抗氧化物毫摩尔浓度，单位为mmol/g。

荧光探针法检测ROS　接种200 μL 10^4 CIK细胞于96孔板(Beaver公司)28 °C、5% CO_2培养箱中培养过夜，设置阴性对照组和tBHQ(10 μmol/L)处理组，每个处理设置5个重复，处理时间点为0、3、6、9、12 h。由于H_2O_2可诱导细胞凋亡造成氧化应激[33-34]，因此该实验使用500 μmol/L H_2O_2作为阳性对照，具体处理步骤：用PBS洗涤培养至12 h的阴性对照和tBHQ处理组，每孔加入200 μL终浓度为500 μmol/L的H_2O_2溶液(PBS稀释)，处理15 min。至相应时间点后，弃去孔内液体，每孔加入100 μL终浓度为10 μmol/L的DCFH-DA (Sigma公司)[26]，28 °C孵育30 min。PBS洗涤3次后，于SpectraMax i3X多功能酶标仪中检测孔板内荧光强度(激发光485 nm，发射光525 nm)。

1.6 tBHQ对*Nrf2*及其下游基因的激活检测

tBHQ对*Nrf2*及其下游基因mRNA表达水平的影响　接种2 mL 10^5CIK细胞于6孔板28 °C、5% CO_2培养箱中培养过夜。设置对照组和tBHQ (10 μmol/L)处理组，每个处理组设置3个重复，时间点为0、3、6、9、12 h。培养至相应时间点后，用RNAiso Plus(TaKaRa公司)试剂收集样品，冻存于–80 °C中保存备用。按上述总RNA提取和cDNA合成步骤提取细胞RNA并合成cDNA。qRT-PCR检测*Nrf2*、*GST*、*HO-1*和*β-actin*的mRNA表达水平。

BHQ对Nrf2蛋白表达水平的影响　接种2 mL 10^5 CIK细胞于6孔板，28 °C、5% CO_2培养箱中培养过夜，设置对照组和tBHQ (10 μmol/L)处理组，每个处理组设置3个重复，时间点为0、3、6、9、12 h。培养至相应时间点后，用RIPA

裂解液(碧云天公司)收集样品，按比例加入5×loading buffer沸水煮15 min，分装保存于−80 °C。按照Nrf2多克隆抗体特异性检测的步骤检验草鱼Nrf2蛋白的组织表达，以*β-actin*作为内参。

1.7　Nrf2在CIK细胞中的定位检测

接种1 mL 10^4 CIK细胞于含爬片的12孔板中，28 °C、5% CO_2培养箱中培养过夜，设置2个对照组和1个tBHQ (10 μmol/L)处理组，每个处理设置2个重复，培养至13 h后，用细胞组织固定液固定细胞20 min，0.3% Triton X-100(Biosharp公司)通透样品25 min，再用山羊血清封闭30 min，Nrf2多克隆抗体和阴性兔血清(1∶400)室温孵育90 min，PBST洗涤4次，每次5 min，FITC荧光二抗(谷歌生物公司)孵育40 min后，同上述步骤洗涤。DAPI细胞核染液(碧云天公司)染色5 min后，同上述步骤洗涤后，将爬片反盖在载玻片上，用Leica Application Suite X共聚焦显微镜拍照观察Nrf2的定位。

2　结果

2.1　草鱼*Nrf2* cDNA的克隆和分析

草鱼*Nrf2* cDNA长1994 bp，其中ORF为1782 bp，编码593个氨基酸(图1)，GenBank登录号为KX243419。ProtParam软件预测出该蛋白分子量为66.50 ku，理论等电点为4.57。Signal IP 4.1 Server程序预测该蛋白不含信号肽。TMpred预测其无跨膜结构。SMART分析结果表明草鱼Nrf2蛋白属于CNC亮氨酸拉链蛋白家族，主要有两个结构域，即PDB结构域(位置在16~39 aa)和BRLZ结构域(位置在483~547 aa)。

利用MEGA 6.0和DNAMAN软件，对草鱼和其他11个物种进行氨基酸序列比对(图2)。结果表明，草鱼Nrf2与其他鱼类的Nrf2相似度比较高，分别是鲤(*Cyprinus carpio*)87%、斑马鱼(*Danio rerio*)83%、墨西哥脂鲤(*Astyanax mexicanus*)68%、兰州鲇(*Silurus lanzhouensis*)65%、大西洋鲑（*Salmo salar*）61%、条石鲷（*Oplegnathus fasciatus*）57%、尼罗罗非鱼(*Oreochromis niloticus*)55%和红鳍东方鲀（*Takifugu rubripes*）52%；与其他动物类群的同源性分别是人46%、鼠45%、鸡47%。另外，该蛋白含有与其他物种Nrf2相似的6个保守功能区Neh1-Neh6、CNC-bZIP区域和7个参与亮氨酸拉链结构形成的保守氨基酸。在系统进化树上，鱼类Nrf2聚在一起，随后和鸡、哺乳类聚在一起，分子进化关系和物种分类关系比较一致(图3)。

2.2　Nrf2多克隆抗体制备及特异性检测

SDS-PAGE分析结果表明，重组的Nrf2蛋白主要表达在包涵体中(图4-a)，因此采用切胶回收的方法提取重组蛋白。用His-tag抗体可以检测到重组的His-Nrf2蛋白只在40 ku左右处有一条带，表明重组蛋白含有His-tag(图4-b)，而且大小和预测的一致，随后用来免疫大耳白兔。用制备的兔多克隆抗体血清进行蛋白免疫印迹检测，结果表明在草鱼CIK细胞蛋白样中可以清晰检测到一条66 ku左右的特异蛋白，大小和预测的Nrf2比较一致。此外，也观察到一条50 ku左右的较弱未知蛋白条带(图4-c)。

2.3　草鱼*Nrf2*的组织表达分析

荧光定量和WB结果表明Nrf2在各组织中均有表达(图5)。脑组织中mRNA表达水平低，和脑组织相比较，其他7个组织中*Nrf2* mRNA相对表达量都是其3.5倍以上，差异极显著(P<0.01)，且在体肾中表达最高，大约是脑组织表达量的24倍(图5-a)。Nrf2蛋白表达水平在脑组织中最低，其他7个组织中的表达水平都是脑组织的3倍以上，差异显著(P<0.05)，且在头肾中表达最高，大约是脑组织中表达量的10倍(图5-b, c)，表明Nrf2组织中mRNA和蛋白水平的表达趋势一致。

2.4　激活剂tBHQ对CIK细胞存活率、总抗氧化能力和ROS的影响

MTT法测得tBHQ对CIK细胞的安全使用浓度为50 μmol/L以下，后续实验使用浓度为10 μmol/L(图6-a)。在处理到6 h时，细胞的总抗氧化能力达到最大值1.09 mmol/g，较0 h差异极显著(P<0.01)；感染6~12 h，细胞的总抗氧化能力较0 h都有显著上调(P<0.05)(图6-b)。

DCFH-DA本身没有荧光，可以自由穿过细胞膜，进入细胞内后，可以被胞内的酯酶水解成DCFH从而将其装载到细胞内。当细胞内产生活性氧时，可以氧化无荧光的DCFH成有荧光的DCF从而反映细胞内的活性氧水平。本实验检测

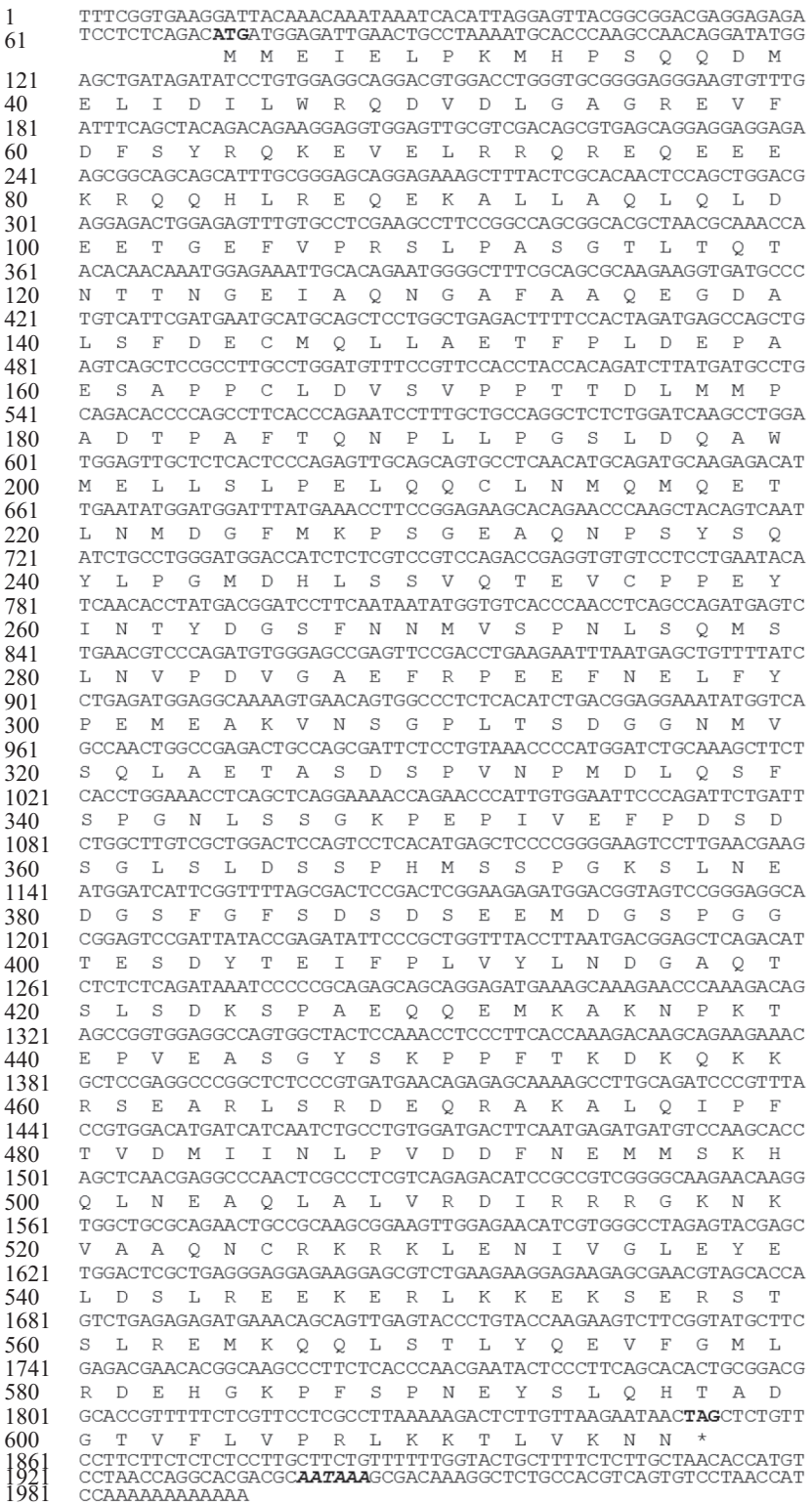


图 1 草鱼*Nrf2*基因cDNA序列及其氨基酸

粗体ATG和TAG分别代表起始密码子和终止密码子，*代表蛋白翻译结束，斜粗体碱基序列AATAAA为加尾信号

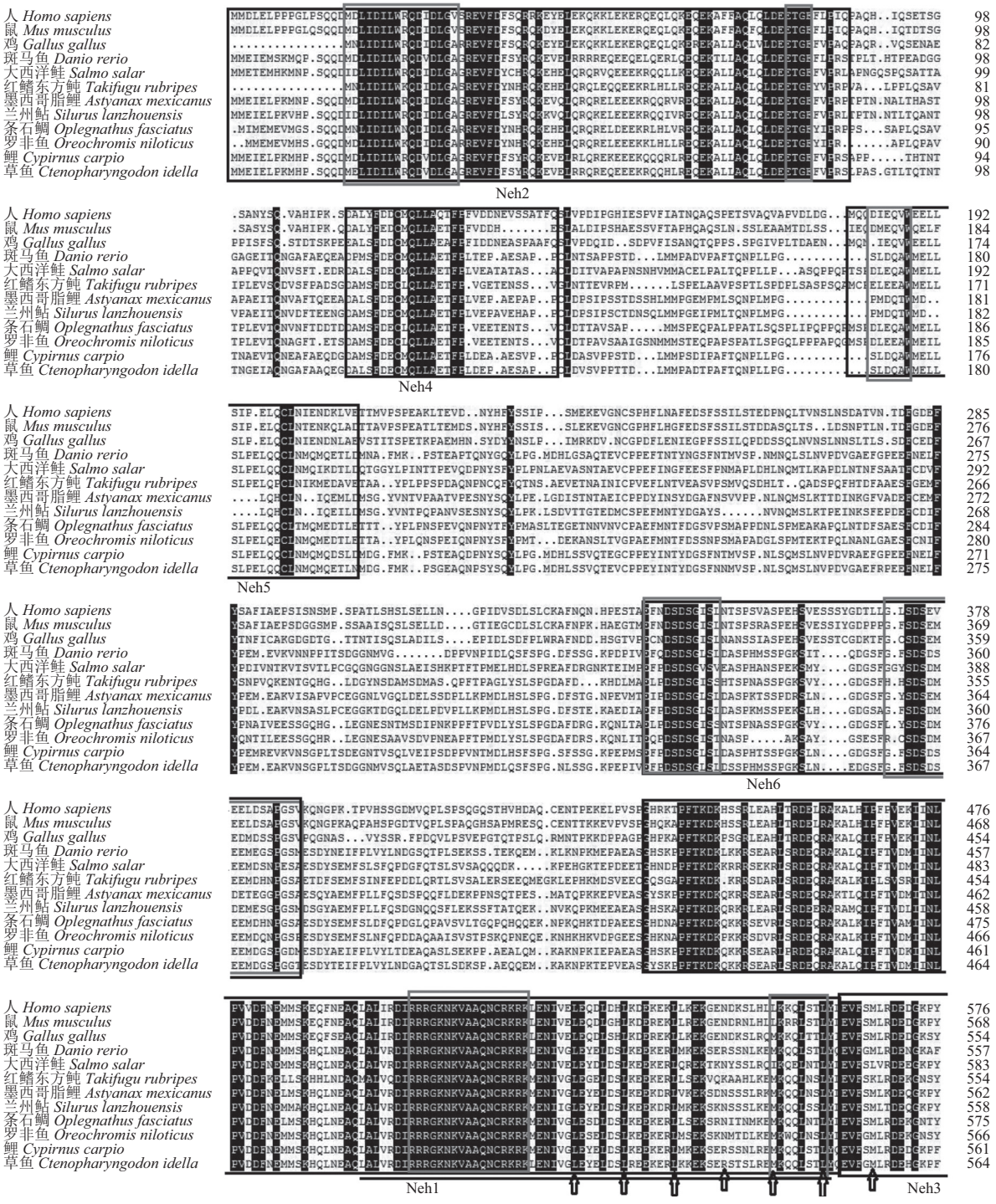
Fig. 1 The cDNA and deduced amino acid sequences of *Nrf2* from *C. idella*

The bold ATG and TAG are the start codon and the stop codon, respectively, the * represents the end of the protein translation, the italic and bold AATAAA sequence is the polyadenylation signal sequence

结果中，其他时间点对照组和处理组的ROS都高于0 h；tBHQ处理CIK细胞3、6、9、12 h后，实验组产生的ROS较对照组都显著下调(P<0.05)，尤其在处理9 h和12 h后，较对照组降低了近1/2。H_2O_2处理组较空白对照组ROS荧光强度上调2.5倍以上，但加入tBHQ后，产生的ROS荧光强度从1.08×10^5降到了8.33×10^4 (图6-c)，表明tBHQ可以抑制CIK细胞活性氧的产生。

2.5　激活剂tBHQ对*Nrf*2及其下游基因的诱导激活

使用激活剂tBHQ处理CIK细胞，随后用荧光定量PCR法检测*Nrf*2、*GST*和*HO*-1的mRNA。结果显示，感染12 h *Nrf2*的mRNA相对表达量较0 h极显著上调(P<0.01)，大约是其7倍(图7-a)；*Nrf*2通路下游基因*GST*和*HO*-1的mRNA相对表达量在9 h时最高，是0 h的6.7倍和13.5倍，随后降低，



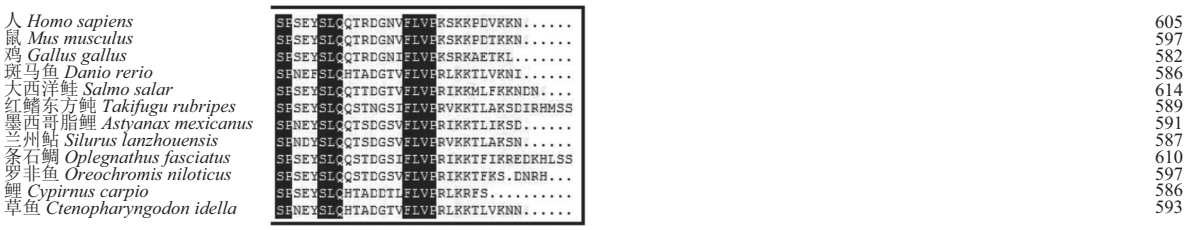


图 2 草鱼与其他物种Nrf2氨基酸多重序列比对

黑色阴影表示一致序列，·表示缺失氨基酸，划线部分为CNC-bZIP区域，黑色方框为Nrf2的6个保守Nrf2环氧氯丙烷同源物(Nrf2-Epoxy chloropropane (ECH) homology, Neh)功能域，灰色方框为Nrf2的功能元件，空心箭头为参与亮氨酸拉链结构形成的保守氨基酸。物种的GenBank ID：人，NP-00615；鼠，NP-035032；鸡，NP-990448；草鱼，KX243419；斑马鱼，AAH45852；大西洋鲑，ACN10720；红鳍东方鲀，ABF22469；墨西哥脂鲤，XP-007258019.1；兰州鲇，ABG90498；尼罗罗非鱼，XP-003447344；条石鲷，BAM36384；鲤，AGI93118.1

Fig. 2 Multiple alignment of Nrf2 amino acid sequences from *C. idella* and other animal species

Amino acids identical in different species were blackened, and the dots (·) represent those that were missing. The cap and collar subfamily basic leucine - zipper (CNC - bZIP) homology region was black line. The six of Nrf2-Epoxy chloropropane (ECH) homology (Neh) domains were black boxes, and gray boxes represent important functional elements within them. The amino acids participating in the putative leucine-zipper dimerization domain were indicated by hollow arrows. The reference sequences were as follows (GenBank ID): *Homo sapiens* (NP_006155), *Mus musculus* (NP_035032), *Gallus gallus* (NP_990448), *Ctenopharyngodon idella* (KX243419), *Danio rerio* (AAH45852), *Salmo salar* (ACN10720), *Takifugu rubripes* (ABF22469), *Astyanax mexicanus* (XP_007258019.1), *Silurus lanzhouensis* (ABG90498), *Oreochromis niloticus* (XP_003447344), *Oplegnathus fasciatus* (BAM36384) and *Cyprinus carpio* (AGI93118.1)

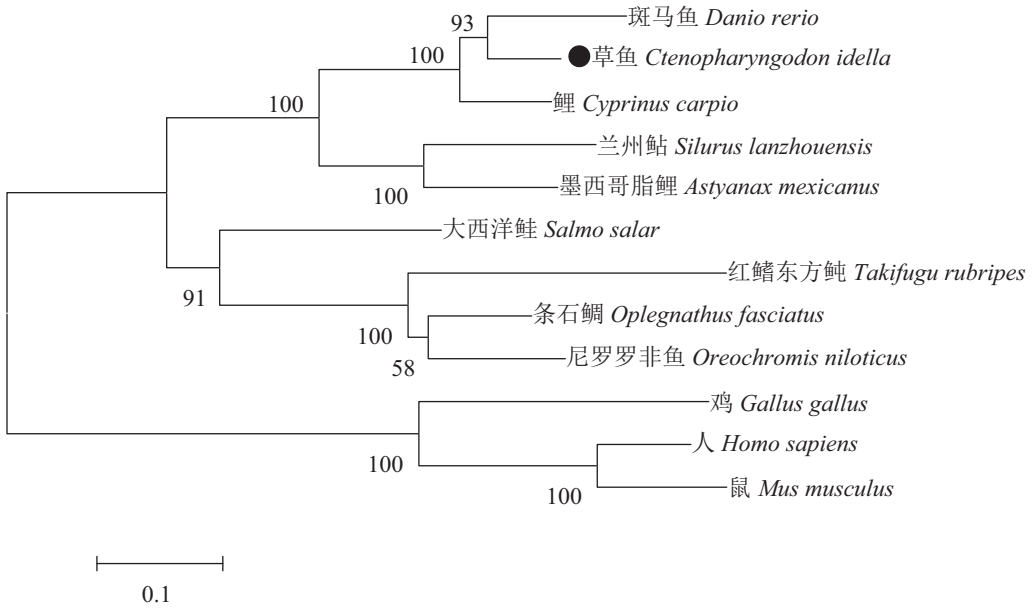


图 3 草鱼与其他物种Nrf2的系统进化树

节点处的数字为1000次引导值中该节点的自举置信水平，圆形标记为草鱼Nrf2

Fig. 3 Phylogenetic tree of Nrf2 from *C. idella* and other animals

The numbers at the branch nodes represent the bootstrap confidence levels of 1000 replication. Nrf2 of *C. idella* was marked in black circle

但都极显著高于0 h时的表达量(P<0.01)(图7-b,c)。WB结果显示，*Nrf2*的蛋白表达量随tBHQ处理时间的增加而上调，且相对表达量差异极显著(P<0.01)(图7-d)。结果表明，当tBHQ处理CIK细胞，抑制其产生活性氧时，诱导了*Nrf2*及其下游的*GST*、*HO*-1基因的表达。

2.6 Nrf2在CIK细胞中的定位检测

共聚焦显微镜观察结果中，阴性兔血清孵育的蛋白样品中没有检测到Nrf2绿色荧光，对照组中可见较弱的绿色荧光，弥散分布在CIK细胞质中；tBHQ处理组中Nrf2的绿色荧光增强，主

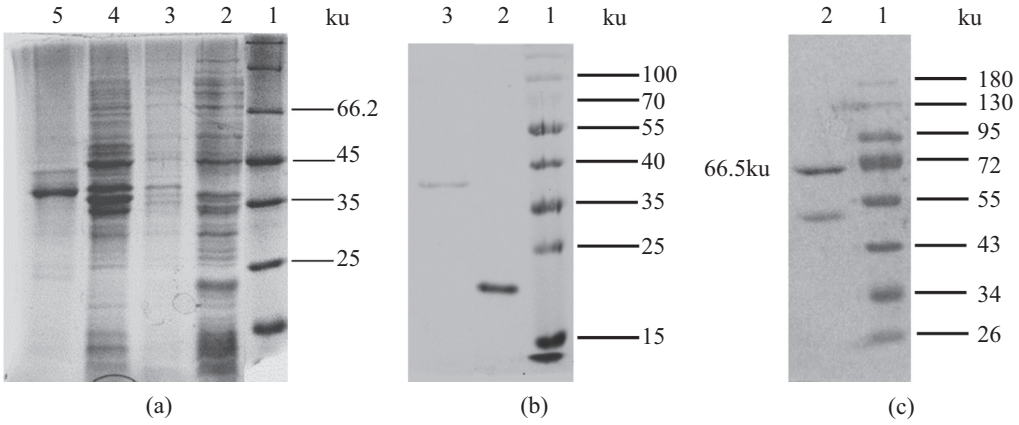


图 4　草鱼Nrf2多克隆抗体制备

(a) SDS-PAGE鉴定纯化的Nrf2重组蛋白，1. 蛋白Marker，2. 表达的pET-32A空载，3. 表达的重组蛋白菌液上清液，4. 表达的重组蛋白菌液沉淀，5. 胶回收样品；(b) His标签一抗检测Nrf2重组蛋白，1. 蛋白Marker，2. 表达的pET-32A空载的总菌体样品，3. 含有重组蛋白的总菌体样品；(c)草鱼Nrf2多克隆抗体特异性鉴定，1. 蛋白Marker，2. CIK细胞总蛋白，大条带分子量为66 ku，和Nrf2大小一致，小条带大小为50 ku左右

Fig. 4　Preparation of polyclonal antibody of Nrf2 from *C. idella*

(a) recombinant Nrf2 protein was purified by SDS-PAGE, 1. protein marker, 2. *E.coli* with pET-32A vector, 3. supernatant of *E.coli* with pET-32A-Nrf2, 4. Pellet of *E.coli* with pET-32A-Nrf2, 5. purified recombinant Nrf2; (b) verification of recombinant Nrf2 protein using His-tag antibody, 1. protein marker, 2. *E.coli* with pET-32A vector, 3. *E.coli* with pET-32A-Nrf2; (c) verification polyclonal antibody of Nrf2, 1. protein marker, 2. total protein of CIK cell, the bigger band was 66 ku which was identical with Nrf2, the smaller band was around 50 ku

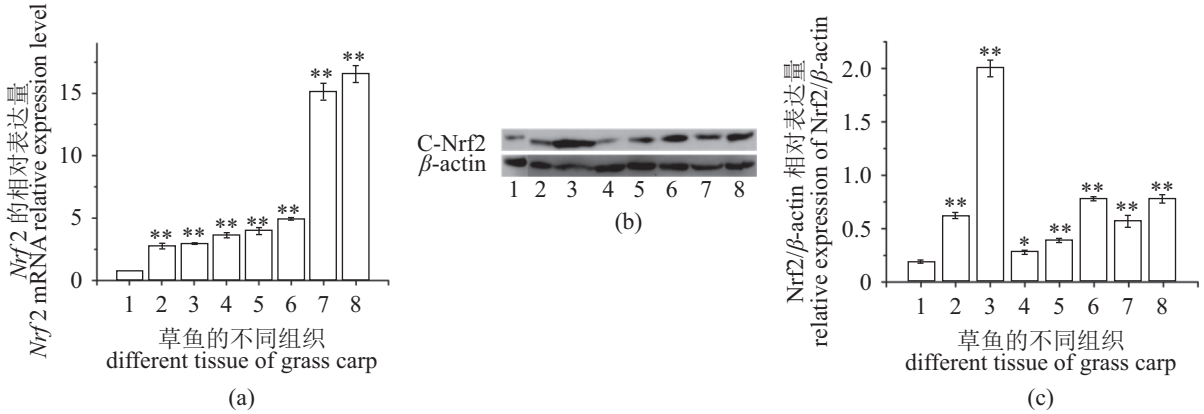


图 5　*Nrf2*基因在草鱼不同组织中的表达

(a) qPCR检测*Nrf2*基因在草鱼不同组织中的mRNA表达(n=5)；(b) WB检测Nrf2蛋白在草鱼不同组织中的表达；(c)柱状图分析(b)图中不同组织Nrf2蛋白相对*β-actin*蛋白条带的灰度值。1. 脑，2. 鳃，3. 头肾，4. 心脏，5. 脾脏，6. 肠，7. 肝脏，8. 体肾。将脑组织Nrf2的mRNA和蛋白质设置为1，作为基数，其他组织的Nrf2数值和它比较。柱上的线条代表平行组的标准误差。*代表显著差异，$P<0.05$；**代表极显著差异，$P<0.01$，下同

Fig. 5　The expression profiles of *Nrf2* in different tissues of *C. idella*

(a) the mRNA of *Nrf2* in the different tissues of *C. idella* by qPCR; (b) the protein of Nrf2 in the different tissues of *C. idella* by WB; (c) histogram analysis of Nrf2β-actin bands in different tissues in Fig (b). 1. brain, 2. gill, 3. head kidney, 4. heart, 5. spleen, 6. intestines, 7. liver, 8. trunk kidney. The Nrf2 mRNA and protein expressed in the brain was set as 1, Nrf2 from all other tissues were compared with that in brain. The bar on column represents that the standard error in parallel group. * represents significant difference, $P<0.05$; ** represents very significant difference, $P<0.01$, the same below

要分布在CIK细胞的核周围，并有少量进入细胞核(图8)。根据核定位结果推测，Nrf2可能通过大量表达和进入细胞核参与调控CIK细胞活性氧的产生。

3　讨论

本实验克隆得到草鱼*Nrf2*基因长1994 bp的cDNA，ORF序列1782 bp，共编码593个氨基酸，

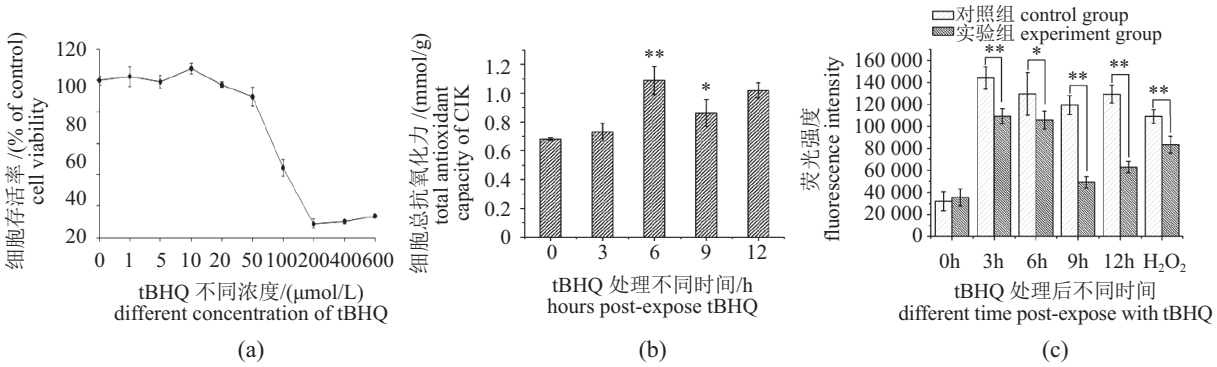


图 6 激活剂tBHQ处理CIK细胞后，对CIK细胞的存活率、细胞总抗氧化能力和细胞内ROS的影响

(a) MTT法检测tBHQ对CIK细胞的存活率的影响；(b)FRAP法检测10 μmol/L tBHQ对CIK细胞的总抗氧化能力的上调作用；(c)荧光探针法检测10 μmol/L tBHQ对CIK细胞产生的活性氧的下调作用，H_2O_2为阳性对照

Fig. 6 The influence on cell viability, total antioxidant capacity and the intracellular ROS of CIK post-treatment by tBHQ activator

(a)the survival rate of CIK treated with tBHQ by MTT methods; (b) up-regulation of total antioxidant capacity of CIK cells treated with tBHQ by FRAP methods; (c) down-regulation of the intracellular ROS of CIK cells exposed to tBHQ by probe methods, H_2O_2 is positive control group

未预测到信号肽序列，这与人、鸡、鼠和斑马鱼的*Nrf*2相似[35-37]。草鱼*Nrf*2基因序列和斑马鱼、鲤的同源性最高，达80%以上，其次是其他几种鱼类，与人、鼠、鸡的同源性最低，只有45%左右，这与物种的进化关系紧密相关。草鱼与斑马鱼、鲤同属鲤科鱼类，而与其他6种鱼类分属不同科，因此同源性有一定的差异；草鱼与人、鼠和鸡的进化跨度较大，因此同源性较低。草鱼Nrf2含有一个位于第483位到第549位氨基酸的CNC-bZip家族的特征序列，表明草鱼Nrf2也是CNC家族的成员[38]。多重序列比对结果中，该蛋白含有Neh1~Neh6 6个保守功能域。Neh1区域含有CNC-bZip结构，在高度保守的碱性区域后，每隔6个氨基酸会出现1个亮氨酸残基，且不同物种不一致；有些位置的亮氨酸残基会被一个极性氨基酸取代，这种结构一方面保证一种CNC蛋白只能专一性识别并结合另一种CNC蛋白，另一方面也防止了同源二聚体的形成[39]。Neh2区域含有DLG和ETGE两个作用位点，可以使该区域和Keap1的BTB结构域结合，从而抑制*Nrf*2的表达[5]。Neh4区域中含有CBP特异的TRAM (transcriptional adapter motif)结合基序(FXEXXXL)，可以单独的激活Nrf2的转录[9]。综上结果表明，草鱼*Nrf*2基因保守性高。*Nrf*2是一个对氧化还原敏感的核相关因子，广泛存在于昆虫、鱼类和哺乳动物中，在肝脏、肾脏和与外界接触频繁的器官中大量表达。也有研究发现，*Nrf*2在人和鼠的肾脏、肌肉、肺、心脏、肝脏和脑中大量表达，肾脏中表达最多，脑组织中表达最少[35, 40]。Yang等[26]用Rt-PCR检测*Nrf*2在鲤肝脏、脾脏、头肾、体肾、鳃、肠和脑7个组织中均有表达，且在体肾和鳃中表达量较高[26]。本研究中通过qRT-PCR和WB发现草鱼*Nrf*2也在多个组织中表达。与其他研究者的结果较为一致，mRNA水平表达量最多的组织是肝脏和体肾，蛋白水平表达最多的组织是头肾。草鱼鳃组织*Nrf*2的表达量和*Nrf*2在鲤鳃组织的表达量不太一致，可能与物种不同有关。草鱼Nrf2组织中的蛋白表达和mRNA表达水平稍有差异，其原因需进一步研究。

Nrf2蛋白预测分子量大约在66 ku，但其在SDS-PAGE胶中出现的位置却具有争议。Moi等[35]研究发现Nrf2的蛋白有66和96 ku两条带；随后Itoh等[36]利用自制的Nrf2抗体也检测到63和97 ku两条带；Venugopal等[41]也检测到66和110 ku两条带[41]；Lau等[42]采用多种方式验证了Nrf2条带在95~110 ku之间。本实验用His-tag一抗确认了重组蛋白含有His-tag。在CIK细胞WB结果中，也检测到2条带，一条表达较强的66 ku条带，和预测的Nrf2蛋白大小一致；另外一条条带较弱，大小约50 ku。本实验获得的结果与之前的研究结果不一致，类似情况也出现在其他转录因子中[40, 43-44]。蛋白不同条带的原因很复杂，和存在剪切异构体、翻译后修饰等有关，需要进一步研究草鱼Nrf2的大小和功能。

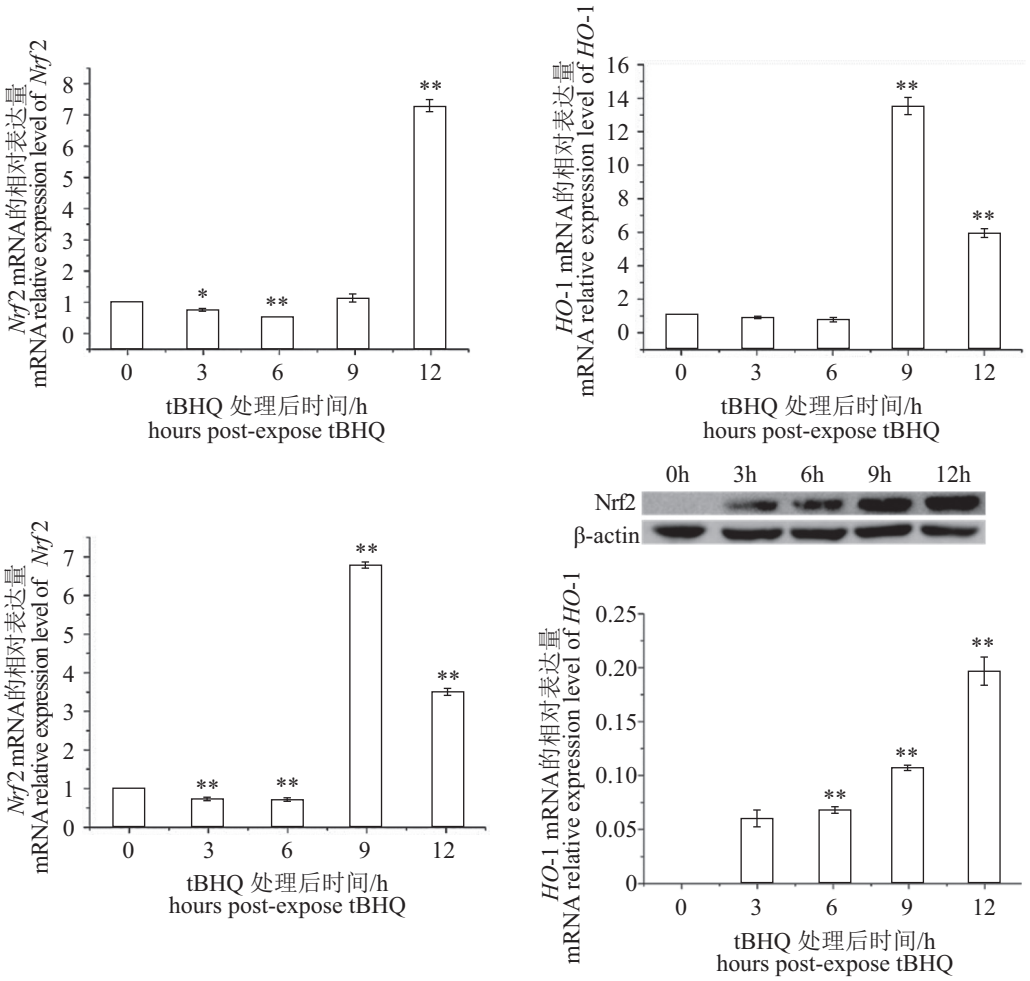


图 7　*t*BHQ激活*Nrf2*及其下游*GST*、*HO*-1基因表达

(a) tBHQ处理不同时间后，CIK中*Nrf2*的mRNA表达水平；(b) tBHQ处理不同时间后，CIK中*HO*-1的mRNA表达水平；(c) tBHQ处理不同时间后，CIK中*GST*的mRNA表达水平；(d) tBHQ处理不同时间后，CIK中Nrf2和β-actin的蛋白表达量及其条带灰度值分析。每个处理设置3个重复，将10^5 CIK细胞接种于6孔板于28 °C 5% CO_2培养箱中培养过夜，翌日用10 μmol/L tBHQ处理0、3、6、9、12 h后，一部分用RANiso Plus溶解样品，提取RNA后用于qPCR分析；一部分样品用RAPI裂解液于冰上裂解20 min后，按比例加入5×loading buffer沸水煮15 min，用于SDS-PAGE和WB分析

Fig. 7　The expressions of tBHQ activated *Nrf2* and its downstream *GST* and *HO*-1 genes

(a) mRNA expression level of *Nrf2* in CIK post exposed to tBHQ at different time points; (b) mRNA expression level of *HO*-1 in CIK post exposed to tBHQ at different time points; (c) mRNA expression level of *GST* in CIK post exposed to tBHQ at different time points; (d) protein expression level of Nrf2 and β-actin in CIK post exposed to tBHQ at different time points and the analysis of gray values of Nrf2/β-actin bands. Every treatment group set in triplicate. We seeded 10^5 concentration CIK cells to six plates and put it in incubator to culture overnight at 28 °C and 5% CO_2. After exposure to 10 μmol/L tBHQ for 0, 3, 6, 9 and 12 h, a part of sample lysis by RNAiso Plus to extract RNA for qPCR analysis. Others lysis by RAPI on the ice for 20 min and then mixed with 5×loading buffer to cook 15 min. The sample analyzed by SDS-PAGE and WB

tBHQ是Nrf2的一种小分子激活剂，能激活Nrf2的核转位，从而特异性上调细胞内抗氧化物质的含量[20]。Nouhi等[45]发现，在小鼠神经退行性疾病中，通过拌饲投喂tBHQ可以激活*Nrf2*的表达，诱导细胞内抗氧化物的增加，从而调控细胞内的氧化应激和凋亡。另外，研究结果表明40 μmol/L tBHQ可以激活褐家鼠PC12细胞和C57BL/6小鼠的*Nrf2*，防止由百草枯和锰引起的神经毒害物质的产生[46-47]。Wu等[48]的研究表明，10 umol/L tBHQ可以激活褐家鼠PC12细胞*Nrf2*的表达从而发挥作用。本实验采用MTT法测定了tBHQ在CIK细胞中的安全使用浓度在50 μmol/L以下，因此采用10 μmol/L tBHQ处理CIK细胞是可行的。

在药物毒性实验的基础上，本研究用FRAP法测定了10 μmol/L tBHQ处理CIK细胞对细胞总抗氧化能力的影响。结果显示经tBHQ处理后，

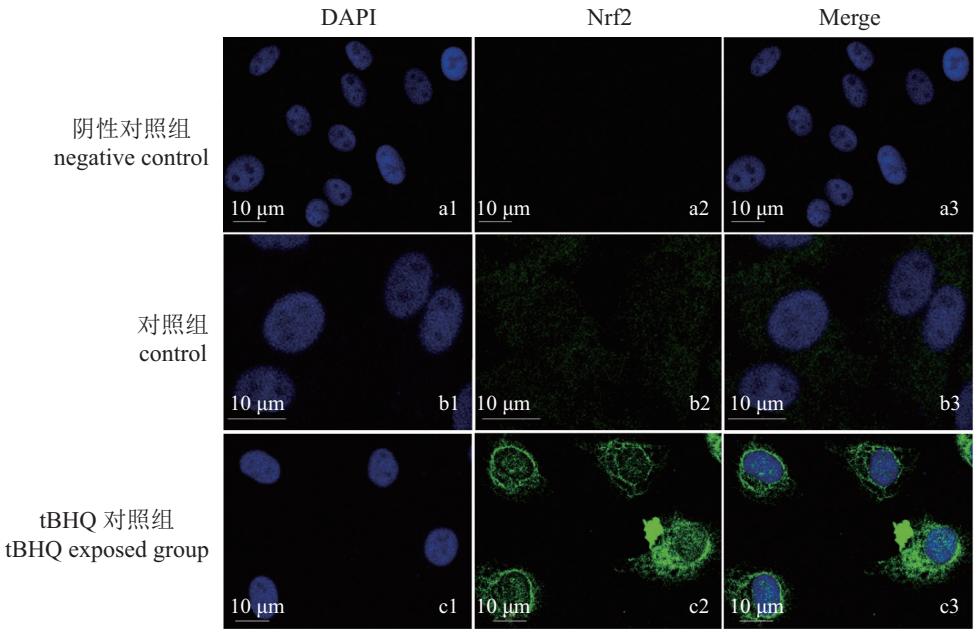


图 8 tBHQ处理CIK细胞后Nrf2蛋白的表达和细胞定位

(a1~a3)阴性兔血清孵育正常CIK细胞；(b1~b3)草鱼Nrf2多克隆抗体孵育正常CIK细胞；(c1~c3)草鱼Nrf2多克隆抗体孵育tBHQ处理过的CIK细胞。用4%的多聚甲醛固定细胞20 min后，0.3% Triton X-100通透25 min。阴性兔血清或者Nrf2多克隆抗体和FITC标记的荧光二抗相继孵育细胞样品1 h后，用DAPI染核。DAPI将细胞核染成蓝色，目的蛋白呈绿色，Merge为合并图片。图片用Leica AF 6000 观察拍照

Fig. 8 The expression and cellular location of Nrf2 in CIK cells post-exposed to tBHQ

(a1–a3) normal CIK cells incubated with negative rabbit serum; (b1–b3) normal CIK cells incubated with polyclonal antibody of Nrf2 from *C. idella*; (c1–c3) tBHQ treated CIK cells incubated with polyclonal antibody of Nrf2 from *C. idella*. After fixed by 4% paraformaldehyde for 20 min, the samples were permeated another 25 min by 0.3% Triton X-100. Samples were incubated with primary antibody or serum from rabbit and goat anti-rabbit IgG (H+L) - FITC successively for 1 h and subsequently stained nucleus using DAPI. C-Nrf2 was green and DAPI was blue. The image were took using Leica AF 6000 Live cell Imaging System

细胞的总抗氧化能力有显著性的上调，推测可能是*Nrf*2激活后，下游抗氧化基因和Ⅱ相代谢酶蛋白的大量表达所致，这有利于提高细胞对ROS的清除能力[49-50]。

呼吸爆发会产生大量的ROS，而ROS的高低是氧化应激发生的标志。ROS主要包含自由基和非自由基氧化分子，如O_2^-、H_2O_2、OH^-。当机体暴露在氧化剂和亲电试剂如环境污染物、烟草、药物和有害异物中，会产生大量的外源性ROS[51]。内源性ROS的产生有很多途径。当ROS作为线粒体中氧化磷酸化的副产物产生时，胞质内的NAPDH氧化酶将会参与调控ROS的产生[52-53]。另外，黄嘌呤氧化酶也参与细胞内ROS的产生[54]。少量表达的ROS对细胞是必不可少的，它参与许多重要的生理功能，如细胞内的信号传导和宿主抵抗微生物。但是细胞内大量ROS的产生会对胞内本身的核酸、脂质和蛋白造成氧化损伤[55]，因此，氧化应激是一把双刃剑，需要精确调控。本研究用荧光探针的方法检测到10 μmol/L tBHQ处理CIK细胞后，细胞内的ROS较对照组有显著地下降，说明tBHQ激活了CIK细胞内某些抗氧化物质的产生，从而使ROS的产生减少，这与Wu等[48]的研究结果一致。

*Nrf*2/ARE是一个重要的参与调控细胞内防御系统的信号通路，通过激活下游一系列抗氧化基因和Ⅱ相代谢酶的表达调控氧化损伤[56]。目前研究发现大约有200个基因受*Nrf*2调控，包括*GST*和*HO*-1[14]。本实验中采用qRT-PCR和WB的方法检测到10 μmol/L tBHQ处理CIK细胞后，*Nrf*2 mRNA水平和蛋白水平表达上调，且其通路下游基因*GST*和*HO*-1的表达也上调，这和其他研究者的结果一致[45, 48, 57]，表明tBHQ可以激活草鱼*Nrf*2及其下游抗氧化基因的表达。

Nrf2的入核受多种因素的影响，如Keap1构象的改变[58]、Nrf2翻译后水平的修饰[59]和泛素化作用[60]。研究表明激活剂可参与迟滞Nrf2的泛素化降解过程[61]。tBHQ拥有一个双酚结构，可以使Nrf2与Keap1解偶联，进入细胞核[62-64]。本实验

通过共聚焦显微镜观察到，用10 μmol/L tBHQ处理后的实验组较对照组，Nrf2的荧光强度增加，且主要聚集在核周围并部分进入细胞核。该结果表明10 μmol/L tBHQ激活了草鱼Nrf2的核转位，与之前的研究结果一致[62-64]。

综上所述，本实验获得了草鱼*Nrf2*基因并制备了有效的兔源多克隆抗体；在草鱼细胞系水平上研究了*Nrf2*及其下游抗氧化基因参与调控细胞ROS的产生，从而调控呼吸爆发过程。对研究呼吸爆发的精准调控在鱼类健康养殖上具有重要意义。

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Cloning and analysis of nuclear factor E2-related factor 2 (*Nrf*2) and its function in the regulation of respiratory burst in grass carp (*Ctenopharyngodon idella*)

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Abstract: Nuclear factor E2-related factor 2 (*Nrf*2) plays a key role in the regulation of respiratory burst which can produce amount of reactive oxygen species (ROS). In the present report, we have cloned and analyzed the *Nrf* 2 gene from grass carp (*Ctenopharyngodon idella*), generated its polyclonal antibody, and characterized its role in regulation of ROS. The obtained cDNA of Nrf2 gene was 1994 bp, containing an open reading frame with 1782 bp encoding 593 amino acids. The amino acid sequences of Nrf2 from grass carp were highly identical with those from common carp (*Cyprinus carpio*) with 87% similarity. Nrf2 from grass carp contained six typical Neh (Nrf2-Epoxy chloropropane (ECH) homology) domains, indicating it was highly conserved in evolution. The qRT-PCR and Western blot analysis showed that Nrf2 was expressed in all eight detected tissues. The total antioxidant capacity of CIKs were up-regulated but ROS generated from CIK cells were down-regulated and the mRNAs of *Nrf*2 and its downstream gene (*GST* and *HO*-1) were all up-regulated when CIK was exposed to the ROS inducer, tert-Butylhydroquinone (tBHQ). Similarly, the expression of Nrf2 protein was also increased and it was translocated in the nucleus of CIK exposed to tBHQ. In conclusion, evolutionary conserved Nrf2 was ubiquitously expressed in the tissues of grass carp and it is involved in down-regulating the generation of ROS in CIK cells via up-regulating the expression of itself and its downstream antioxidant genes, so as to regulate respiratory burst.

Key words: *Ctenopharyngodon idella*; NF-E2-related factor 2; gene cloning; polyclonal antibody; ROS

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