Mapping of ATP, Glucose, Glycogen, and Lactate Concentrations Within the Arterial Wall

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Objective—In large- and medium-sized arteries, the diffusion distances for oxygen and nutrients are long. This has been suggested to make these vessels prone to develop local energy metabolic deficiencies that could contribute to atherosclerosis. To validate this hypothesis, we introduced a new method to measure energy metabolites within the arterial wall at high spatial resolution.

Methods and Results—Bioluminescence imaging was used to quantify local metabolite concentrations in cryosections of snap frozen (in vivo) and incubated pig carotid artery rings. Incubation at hypoxia resulted in increased lactate concentrations, whereas ATP, glucose, and glycogen concentrations were decreased, especially in the mid media, where concentrations of these metabolites were close to zero. In snap frozen arteries, glycogen concentrations were markedly higher in deep layers of the media than toward the lumen. ATP, glucose, and lactate were more homogenously distributed.

Conclusions—Bioluminescence imaging is a new and powerful tool to assess arterial wall energy metabolism at high spatial resolution. Our experiments demonstrate heterogeneous distributions of energy metabolites under hypoxic in vitro conditions. Furthermore, we show that glycogen concentrations are higher in deep medial layers in vivo. This might represent a local adaptation to a low-oxygen microenvironment. (Arterioscler Thromb Vasc Biol. 2003;25:1801-1807.)

Key Words: atherosclerosis ■ hypoxia ■ bioluminescence ■ energy metabolism ■ imaging

The arterial wall is supplied with oxygen and nutrients by diffusion and convection from luminal blood and from vasa vasorum in the adventitia and outer media. Metabolic waste products are removed by diffusion in the opposite direction. Diffusion distances in large- and medium-sized arteries are often close to, or even exceed, the 100 to 200 μm that is frequently stated as the diffusion limit for oxygen.1–4 Based on these facts, it has been suggested that the arterial wall is predisposed to develop local energy metabolic disturbances and that this is a key factor both in the initiation and progression of atherosclerosis.1 In atherosclerosis-prone areas, both insufficient transport6,7 and increased consumption of metabolites8 could contribute to local disturbances. Later in the disease process, increased diffusion distances, attributable to intimal thickening as well as a high consumption of oxygen8 and glucose9,10 by foam cells, could additionally aggravate energy metabolic predicaments and contribute to the development of a necrotic core. Supportive of this hypothesis, hypoxic zones have been demonstrated in situ in the normal pig arterial wall11 as well as in vitro12–14 and in vivo15 in rabbit atherosclerotic plaques. However, ATP production may be maintained under hypoxic conditions via anaerobic breakdown of glucose and glycogen, but this could lead to a potentially harmful accumulation of lactate. Therefore, other aspects of energy metabolism besides the supply of oxygen need to be analyzed. To the best of our knowledge, bioluminescence imaging is the only method available that allows analysis of ATP, glucose, and lactate concentrations at the necessary spatial resolution.16 We have recently adapted this method to measure local ATP concentrations within the arterial wall.14 In the present study, our methodology is extended to include determinations of glucose, glycogen, and lactate. The use of bioluminescence imaging to measure glycogen concentrations is presented for the first time in this article.

The high spatial resolution of bioluminescence imaging allows assessment of the energy metabolic situation at defined histological locations in different parts of the arterial tree. It is in essence an in vitro method, but it may be used to reflect the in vivo situation when snap frozen tissue is analyzed. It thus constitutes a new and powerful tool that for the first time allows concentrations of arterial wall energy metabolites to be measured at a microscopic level.

Methods

Snap Freezing and Incubation of Tissue

Experiment Set Up

Methodological Experiments

A series of methodological experiments was conducted to optimize measurement conditions. The main aspects of these experiments are...
described in the online supplement, available at http://atvb.ahajournals.org.

Snap Frozen Tissue: In Vivo
Snap frozen pig carotid arteries were used to evaluate the in vivo situation. The same tissue was also used to analyze the methodological variation of bioluminescence imaging.

In Vitro Experiments
Two different sets of in vitro experiments were performed where pig carotid arteries were exposed to hypoxia and glucose deprivation at varying degree. In the first set of experiments, incubation was performed for 3 hours. The purpose of these experiments was to study the relation between energy metabolic status (ATP), the availability of energy substrates (glucose and glycogen), and the production of waste product (lactate) under varying oxygen and glucose concentrations. In the second set of experiments, substrate-free medium and varying incubation times (0 to 6 hours) were used. The purpose was to study the glycolysis breakdown rate under various oxygen concentrations and relate this to the energy metabolic status (ATP) in the tissue.

Animals
The study protocol was approved by an Animal Ethics Committee and conducted in accordance with NIH guidelines for the use of experimental animals. Carotid arteries were obtained from domestic pigs (51 to 74 kg). The animals were premedicated with 4:1 ketamine (Ketalar 100 mg/mL, Parke-Davis) and midazolam (Dormicum 5 mg/mL, Roche). After intubation, anesthesia was induced with 0.07 to 0.14 mL of thiopental sodium IV (pentothal natrium 60 mg/mL, Abbott). After intubation, anesthesia was maintained with isoflurane (Isofluran Pharmacia, Pharmacia & Upjohn) in N₂O/O₂.

The right carotid artery was surgically exposed, and 1 cm-long segment was rapidly snap frozen in liquid nitrogen. Freezing was done within 15 seconds from induction of cardiac arrest (KCl IV) and within 2 to 3 seconds from removal. An additional 6 cm-long segment to be used for in vitro incubations was put into oxygenated (75% O₂, 5% CO₂ in N₂) PBS (without calcium and magnesium) with 5.6 mmol/L glucose.

Medium
PBS (without calcium and magnesium) with 25 mmol/L HEPES and 10 mmol/L bicarbonate with 0 or 5.6 mmol/L glucose was used throughout.

Dissection and Incubation
In the 6 cm segments used for in vitro studies, the adventitial tissue was carefully removed by dissection in circulating oxygenated (75% O₂, 5% CO₂ in N₂) PBS at 37°C, and the vessel was divided into 5-mm rings and distributed into incubation vials.

In the first set of experiments (3 independent experiments), arterial segments were subjected to different degrees of energy metabolic restriction by incubation for 3 hours at different oxygen (75% or 3%) and glucose (5.6 or 0 mmol/L) concentrations at 37°C. In the second set of experiments (2 independent experiments), arterial segments were allowed to exhaust their glycogen stores by incubation in glucose-free PBS at 75% or 3% oxygen for different time periods (0, 0.5, 1.5, 3, and 6 hours) at 37°C.

Tissue Preparation
Immediately after surgical removal or incubation, the aortic rings were snap frozen in liquid nitrogen, mounted in OCT (Tissue-Tek) and stored at −70°C until further use. From each ring, 24 consecutive cryosections (10 μm) (Leica cryostat at −25°C) were mounted on poly-l-lysine slides (Sigma). The sections were immediately fixed on a heating plate (95°C) for 10 minutes and then stored at −20°C until analysis. Additional 10-μm sections were taken from 10 artery rings (5 snap frozen, 5 from in vitro experiments). These sections were air dried overnight and subsequently stained with Mayer’s hematoxylin and used to determine cellularity profiles.

Cellularity Profiles
The cell nuclei in 10-μm sections from 10 artery rings were stained with Mayer’s hematoxylin. From each ring, digital images of 4 lumen to adventitia profiles (at 3, 6, 9, and 12 o’clock) were obtained. Each profile was a composite of 3 to 4 visual fields with a ×40 objective (Zeiss Axiosplan 2 microscope and an AxioCam cooled CCD color camera, Carl Zeiss). The profiles were divided into 100-μm-thick zones based on the distance from the lumen and the areas, and number of nuclei in each of these zones was calculated (KS 400 software, Carl Zeiss).

Bioluminescence Imaging
Bioluminescence imaging was used to visualize and quantify the distribution of metabolites within cryosections. A range of methodological experiments was performed to optimize spatial resolution and the quantitative calibration as well as to define methodological variation of this method. A description of these experiments as well as detailed methodological instructions are given in the online supplement. A brief methodological description is given below.

Measurement Procedure
An enzyme solution containing luciferase is applied (in complete darkness) to a cryosection on a microscope stage. This leads to emission of photons in proportion to the concentration of the studied metabolite (ATP, glucose, glycogen, or lactate). The photons are registered by a photon-counting camera (C2400-47, Hamamatsu Photonics) mounted on the microscope (Axiovert 135 M, Carl Zeiss). The light intensity (gray value) in different parts of the resulting digital bioluminescence image reflects the local metabolite concentrations. A darkfield image of the same section is also obtained to outline histological structures of the corresponding bioluminescence image. From every artery ring, 5 or 6 consecutive sections were analyzed for each of the 4 metabolites. Each section was used for the analysis of 1 metabolite, and all measurements were performed at room temperature (23±1°C).

Standards
To calibrate the bioluminescence signal, standards were made by dissolving different concentrations of ATP, glucose, glycogen, and lactate in physiological saline with 8% low-molecular-weight gelatin. The solution was frozen, and 10-μm cryosections were made and treated in the exact same way as the tissue sections.

Image Analysis
To obtain values for the mean metabolite concentration within the whole media (ie, global metabolite concentration), the mean bioluminescence intensity (gray value) in 5 to 6 consecutive sections was calculated. To evaluate differences in metabolite concentrations at different distances from the lumen, concentration profiles were determined. Each profile is an average of the bioluminescence intensity along 12 virtual sections at 3, 6, 9, and 12 o’clock in 3 consecutive sections. Areas where small air bubbles had been trapped in the enzyme solution were excluded from measurement. KS 400 software (Carl Zeiss) was used throughout.

Determination of Methodological Variation
Snap frozen pig carotid artery segments from 5 animals were used to define methodological variation. From every artery segment, 24 sections were analyzed, ie, 6 each for the metabolites ATP, glucose, glycogen, and lactate.

Variability Between Consecutive Sections
Variation in bioluminescence intensity between measurements on 6 consecutive sections was analyzed in each of the 5 artery segments (ie, 5×6=30 sections per metabolite).

Variability Within Single Sections
All metabolites showed a homogenous distribution pattern along the circumference of the artery. Therefore, variation within sections could be assessed by determining mean metabolite concentrations in the 4 analog segments of each section (ie, 5×6×4=120 quadrants per metabolite).
Statistics

Coefficients of variation and global metabolite concentrations are expressed as mean±SD. ANOVA with Bonferroni correction was used to compare groups in Figure 3, and \( P<0.01 \) was considered significant.

Results

Methodology

Methodological Variation

Consecutive sections from snap frozen segments of pig carotid artery (n=5) were used to assess methodological variability. The coefficient of variation between repeated measurements on consecutive sections was \( \approx 10\% \) for ATP (10±2%) and lactate (10±3%), whereas it was approximately twice as high for glucose (18±3%) and glycogen (20±5%). The variation within analog segments of a single section was lower, and the coefficient of variation ranged from 7±4% for ATP and lactate to 12±6% for glycogen (glucose, 9±5%).

Figure 2. Cellularity profile. The number of cells (mean±SD) per square millimeter at different distances from the lumen in pig carotid arteries (n=10). Cellularity was approximately 20% lower toward the adventitia than close to the lumen.

Details of the content require careful reading and understanding of the experimental design and statistical analysis. The key findings include:

- The ATP- and glucose- and glycogen-dependent bioluminescence intensity in 10-μm frozen sections with known metabolite concentrations is shown in Figure 1. The standard curves were subsequently used to transform bioluminescence intensity (relative units) in tissue sections into actual metabolite concentrations (micromolar per gram of wet weight).

- Cellularity profiles were determined to exclude the possibility that different metabolite concentrations were attributable to differences in cellularity between different medial layers. Cellularity was \( \approx 20\% \) lower toward the adventitia (2100±200 cells/mm²) than toward the lumen (2500±240 cells/mm²) (Figure 2). There was no difference in the cellularity profiles between snap frozen arteries and arteries that had been used for in vitro experiments.

- The global metabolite concentrations in snap frozen pig carotid arteries (n=5) were analyzed.

- Global Metabolite Concentrations

The global metabolite concentrations in snap frozen pig carotid arteries (n=5) are given in Figure 3 (black bars).
Global Concentrations

Glucose concentrations were reduced to 63% and 49% when glucose was present during incubation, it is likely that the ATP production in this set up, at least in part, derived from breakdown of glycogen. When both oxygen and glucose supply were compromised, there was a pronounced reduction of ATP to 9.4±9.0%, glucose to 3.6±3.2%, and glycogen to 0.5±3.1%. Lactate was reduced to 53±18%. Thus, glycogen breakdown was more pronounced under hypoxic conditions, and depletion of available stores coincided with failed ATP production.

Concentration Profiles

Concentration profiles were assessed to detect heterogeneities in the concentration of metabolites at different depths of the tissue (Figure 4). The concentration profiles indicated homogenous ATP and lactate concentrations throughout the media. Glucose concentrations, however, were higher toward the lumen, declined to reach a nadir in the mid media (≈400 to 500 μm from the lumen), and increased again toward the adventitia. Glycogen distribution showed an opposite pattern, with higher concentrations in the mid media than toward the lumen and adventitia.

In Vitro

Effect of Incubation at Different Oxygen and Glucose Concentrations

Pieces of normal pig carotid artery were incubated for 3 hours at 37°C in medium with different oxygen (75% or 3% O2) and glucose (0 or 5.6 mmol/L) concentrations.

Global Concentrations

To emphasize changes, ATP, glucose, glycogen, and lactate concentrations are given in percents, with 100% corresponding to concentrations after incubation at 75% O2 and 5.6 mmol/L glucose. The absolute concentrations, in micromolar per gram of wet weight, are given in Figure 3. When oxygen supply was compromised (3% O2) at 5.6 mmol/L glucose in the medium, ATP concentrations were reduced to 49±7%. Glucose concentrations were reduced to 63±27%, and glycogen concentrations to 33±14%. Lactate concentrations, however, were increased to 145±32%. Thus, ATP concentrations were reduced by approximately half, despite the indication of an increase in anaerobic breakdown of glucose and glycogen to lactate under hypoxic conditions. When glucose was removed from the medium but oxygen supply was abundant, ATP concentrations were slightly reduced to 88±16%, and glycogen and lactate concentrations were reduced to 49±14% and 59±22%, respectively. As expected, glucose concentrations were very low, reduced to 5.1±4.7%. The ATP production in the absence of extracellular energy substrates can be maintained by utilization of intracellular energy stores, eg, glycogen or fat. Because glycogen concentrations were approximately 50% lower than

Concentration Profiles

Determination of concentration profiles made it possible to detect heterogeneities in the concentration of metabolites at different depths of the tissue. At high oxygen and 5.6 mmol/L glucose, there was a glucose gradient with an approximately 20% lower glucose concentration in the mid media (≈400 μm from the lumen). Glycogen concentrations, on the other hand, were lower in the layers adjacent to the lumen than toward the adventitia. ATP and lactate were more homogeneously distributed (Figure 5). Reduction of the supply of oxygen with glucose concentrations maintained had a pronounced effect on the concentration profiles. The concentrations of ATP, glucose, and glycogen were zero in the mid media, approximately 300 to 400 μm from the lumen, and higher toward lumen and adventitia. Thus, local substrate depletion in the mid media colocalized with failed ATP production. However, lactate concentrations were increased throughout the media and were not heterogeneously distributed (Figure 5). With abundant oxygen but without glucose, the ATP and glycogen concentrations were slightly higher toward the adventitia than in the luminal layers. Lactate and glucose concentrations were low, and these metabolites were more evenly distributed within the tissue (not shown). Reduction of the supply of oxygen (3%) as well as glucose resulted in ATP depletion in medial layers deeper than >100 to 200 μm from the lumen. However, in a 100- to 200-μm-wide zone adjacent to the lumen, ATP concentrations were higher, up to 0.10 μmol/g wet weight. Glucose, glycogen, and lactate concentrations were low and more homogeneously distributed (not shown).
Effect of Incubation Without Energy Substrates for Different Time Periods

To exhaust the available intracellular glycogen stores, pieces of pig carotid artery were incubated in well-oxygenated (75% O₂) or hypoxic (3% O₂) glucose-free PBS for 0.5 to 6 hours. Zero hours (t=0) represents an artery segment frozen after dissection, immediately before incubation.

In incubations with high oxygen (Figure 6, solid lines), ATP concentrations were only slightly reduced after 3 hours. After 6 hours, however, ATP concentrations were close to zero. Glycogen concentrations showed a gradual decline and were very low after 6 hours. Glucose and lactate concentrations were reduced at all time points. In comparison with high-oxygen incubations, hypoxia (Figure 6, dashed lines) resulted in very low concentrations of ATP as well as glycogen already after 1.5 hours. Lactate concentrations, on the other hand, were higher after 0.5 hours but at a similar level as in well-oxygenated medium after prolonged incubation. Glucose concentrations were reduced at all time points and thus were not influenced by oxygen concentrations. The data indicate an anaerobic breakdown of glycogen under hypoxic conditions, with a more rapid depletion of available stores and subsequent failure of ATP production.

Discussion

In a recent publication, we introduced a new technology to determine ATP concentrations within the arterial wall at high spatial resolution. In this study, our methodology is extended to include determinations of local glucose, glycogen, and lactate concentrations as well. Both snap frozen (in vivo) and in vitro incubated pig carotid arteries were analyzed. The results presented here lend themselves to 2 principally different discussions. First, the new methodology can detect local heterogeneities in the concentration of energy metabolites within the arterial wall. Second, the in vivo and in vitro data from our experiments add new insight into the heterogeneity of energy metabolism within the arterial wall.
Methodology
The methodology is based on the registration of metabolite-specific bioluminescence in frozen tissue sections. In this study, the technique was used to determine global metabolite concentrations (average concentration in the whole media) and concentration profiles within the arterial wall. A range of established techniques can be used to measure global concentrations (ie, data as in Figures 3 and 6) of energy metabolites. However, these methods do not provide any information about the regional distribution. The analyses performed in this study illustrate the importance of these regional assessments, because the metabolite distributions might well be heterogeneous (Figures 4 and 5). The variability in bioluminescence intensity between analogous segments of single sections is low (coefficient of variation, 7% to 12%), which makes registration of false heterogeneities unlikely. If consecutive sections are used for the analysis of ATP, glucose, glycogen, and lactate, these key aspects of energy metabolism can be studied within the same microscopic location (Figures 4 and 5). An additional strength is that additional sections may be used for histological staining, immunohistochemistry, and in situ hybridization.

Inherent to the presented method is diffusion of substrate from the frozen sections during bioluminescence registration.14,17 Optimization of measurement conditions (described in the online supplement) has reduced this artifact, but true tissue gradients will be slightly underestimated. This is most clearly illustrated at tissue borders, where an infinitely steep gradient would be expected but a less steep gradient is registered (profiles in Figures 4 and 5).

To get a true reflection of the in vivo situation with the presented methodology, the time from cessation of blood flow until the tissue is frozen should be minimized. During the delay between removal and freezing, metabolite concentrations could change, eg, ATP would be expected to decline.18 Furthermore, diffusion of small solutes before freezing could lead to smoothening, and thus underestimation, of true tissue gradients. In our study, the time from cessation of blood flow to snap freezing was kept at an absolute minimum (between 2 to 3 seconds), and we believe that our results reflect the in vivo situation. However, it is impossible to rule out that in vivo metabolite concentrations could be subject to some change even when freezing is almost instantaneous.

Aerobic Lactate Production
The lactate determinations performed in our study indicated a substantial lactate production even after incubation in an excess of oxygen, where more efficient oxidative metabolism would have been expected to occur (Figures 3 and 5). Glucose, and not glycogen, seemed to be the main substrate for this aerobic lactate production, because without glucose, lactate concentrations were lower despite remaining glycogen stores (Figure 3). Our findings support previous in vitro studies claiming that aerobic glycolysis is a characteristic feature of vascular smooth muscle cells.19–21 and that glucose, and not glycogen, is the substrate used in this process.19 As an explanation of this phenomenon, it has been suggested that different metabolic pathways are compartmentalized within the vascular smooth muscle cells and that glycolysis is specifically coupled to sodium and potassium transport processes whereas oxidative metabolism is coupled to contractile energy requirements.19 This compartmentation, which is not absolute,22,23 has been suggested to lead to a more efficient coupling between energy supply and transduction. The finding that lactate concentrations were even higher in snap frozen tissue (Figures 3 and 4) strongly suggests that the high lactate concentrations were not an in vitro artifact but rather a reflection of normal vascular smooth muscle metabolism.

Energy Metabolism Under Hypoxia
When the availability of oxygen in the surrounding medium was compromised, adequate ATP production could not be maintained. Glucose and glycogen concentrations were decreased and lactate concentrations increased, indicating a compensatory increase in anaerobic metabolism (Figures 3 and 5), in agreement with other studies.21,24 This seemed to offer a certain degree of protection against hypoxia-induced energy metabolic failure, because ATP concentrations were much lower at low oxygen without glucose (Figure 3). In the mid media, ATP, glucose, and glycogen concentrations were close to zero (Figure 5). Identical in vitro conditions have previously been shown to induce severe hypoxia in this zone of the normal rabbit aorta.13,14 The pig carotid artery is approximately twice as thick as the rabbit aorta and would be expected to be close to anoxic in the mid media under the same experimental conditions. The shortage of oxygen would be expected to lead to an increase in anaerobic energy metabolism, consequently straining the available diffusion capacity for glucose as well as breakdown of the intracellular glycogen stores. Thus, the mid medial ATP depletion is probably secondary to the local deficiency of both oxygen and substrates in this zone.

A decrease in arterial wall oxygenation attributable to atherosclerosis,25,26 hypertension,27 or hypercholesterolemia28 leads to a compensatory increase in the number of adventitial vasa vasorum. This increases tissue perfusion and counteracts the development of arterial wall hypoxia in vivo.29 However, there seem to be a limit for this compensatory mechanism, because the core of rabbit atherosclerotic plaques is severely hypoxic in vivo when the intimal thickness exceeds 300 μm.15 This failure to adequately increase tissue perfusion might be explained by the more irregular growth pattern and poorly supported wall of the newly formed vasa vasorum.26,28

Glycogen as an Energy Substrate
The relative importance of different intracellular energy stores in vascular smooth muscle cells is controversial, and varying glycogen stores have been reported from different vessels.29–31 In this study, we show that ATP levels could be maintained without exogenous energy substrates, but only when sufficient glycogen stores were available (Figures 3 and 6). Based on data from independent groups, it has previously been estimated that glycogen stores can maintain ATP production for 38 minutes in contracting pig carotid arteries.32 However, in our setup, ATP levels were maintained for 3 hours under well-oxygenated conditions. This might be explained by the fact that the vessel segments were unstimulated in our experiments and could thus be expected to have
a substantially lower energy demand than contracting arteries. Glycogen depletion was more rapid and the initial lactate concentrations were higher under hypoxic conditions (Figure 6). This strongly suggests an anaerobic, and less-efficient, breakdown of glycogen under hypoxic conditions and, as a result, a more rapid depletion of the available glycogen stores.

Glycogen concentrations in snap frozen carotid arteries were higher in the deep layers of the media than close to the lumen (Figure 4). Consequently, glycogen concentrations were high in the layers where in vivo oxygen concentrations would be expected to be low.11 It has recently been shown that culture of rat tail arteries under hypoxic conditions leads to a very pronounced upregulation of the glycogen stores.29 A possible explanation of our finding could thus be that the hypoxic microenvironment in the mid media leads to a local upregulation of the glycogen stores in these layers. This adaptive mechanism could make these layers more tolerant to future episodes of hypoxia and nutrient shortage.

Conclusion
Local energy metabolic disturbances within the arterial wall have been proposed to be a key factor in atherogenesis. In this study, we introduce a novel methodology that allows concentrations of arterial wall energy metabolites to be measured at a microscopic level. With this new technique, pronounced heterogeneities in the concentrations of ATP, glucose, and glycogen under hypoxic in vitro conditions were demonstrated. Furthermore, we show for the first time that glycogen concentrations in pig carotid arteries are higher in deep medial layers than close to the lumen. The upregulation of glycogen stores in these layers might represent an adaptation of the smooth muscle cells to a low-oxygen microenvironment.

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**Materials and methods – supplementary data on line**

**Bioluminescence imaging**
Bioluminescence imaging \(^1\text{-}^3\), was used to visualize and quantify the distribution of metabolites (ATP, glucose, glycogen, and lactate) within cryosections. In short, an enzyme solution containing luciferase is applied to a cryosection on a microscope stage. This leads to emission of photons in proportion to the concentration of the studied metabolite and the photons are registered by a photon counting camera mounted on the microscope. The light intensity in the different parts of the cryosection reflects the local metabolite concentration.

**Enzyme solutions**
To link the substrates of interest to the production of photons, different enzyme solutions were used. The biochemical background for the bioluminescence reactions are outlined in online Figure I.

**ATP dependent bioluminescence**
Enzyme solution for ATP dependent bioluminescence was prepared by dissolving powderized firefly lanterns (Sigma) in 0.1 M Arsenate buffer with 0.2 M HEPES (pH 7.6) and 40 mM MgCl\(_2\). The procedure has been described in detail elsewhere \(^2\).

**Glucose dependent bioluminescence**
To 1000 µl of 0.3 M PBS with ATP (200 mM), MgCl\(_2\) (13 mM), 1,4-dithiothreitol (DTT) (8.2 mM), flavin mononucleotide (FMN) (1.6 mM) and capric aldehyde (13 mM) (pH 7.0); a co-factor (NADP) and enzymes were added in the amounts given below:

- 500 µl of 0.3 M NADP in 0.3 M PBS with 0.3 M HEPES.
- 60 µl of hexokinase (1500 U/mL) in 0.3 M PBS. To prepare this solution, 1 mL hexokinase in 3.2 M ammonium sulfate solution was centrifuged in an eppendorf centrifuge for 1 minute. After centrifugation, the supernatant was removed and the pellet carefully dissolved in 1 mL 0.3 M PBS.
- 60 µl of glucose-6-phosphate dehydrogenase (1750 U/ml) in PBS. Glucose-6-phosphate dehydrogenase in PBS was prepared exactly as hexokinase in PBS (see above).
- 180 µl of luciferase from photobacterium fischeri (200 mU/mL) in 0.3 M PBS.
- 200 µl of NADPH-FMN-oxireductase (130 U/mL) in 0.3 M PBS.

During preparation, all chemicals were kept on ice and in subdued light (flavin mononucleotide is light sensitive). When the enzymes and cofactors had been dissolved in the buffer, the pH was set to 7.0. Enzymes and NADP were from Roche, the other chemicals were from Sigma.

**Glycogen dependent bioluminescence**
To 1000 µl of 0.3 M PBS with ATP (200 mM), MgCl\(_2\) (13 mM), 1,4-dithiothreitol (DTT) (8.2 mM), flavin mononucleotide (FMN) (1.6 mM) and capric aldehyde (13 mM) (pH 7.0); a co-factor (NADP) and enzymes were added in the amounts given below:

- 500 µl of 0.3 M NADP in 0.3 M PBS with 0.3 M HEPES.
- 740 µl of phosphoglucomutase solution (1650 U/mL) was centrifuged in an eppendorf centrifuge for 1 minute. After centrifugation, the supernatant was removed and the pellet carefully dissolved in the enzyme solution.
- 60 µl of glucose-6-phosphate dehydrogenase (1750 U/ml) in PBS. To prepare this solution, 1 mL glucose-6-phosphate dehydrogenase in 3.2 M ammonium sulfate solution was centrifuged in an eppendorf centrifuge for 1 minute. After centrifugation, the supernatant was removed and the pellet carefully dissolved in 1 mL 0.3 M PBS.
- 180 µl of luciferase from photobacterium fischeri (200 mU/mL) in 0.3 M PBS.
• 200 µl of NADPH-FMN-oxireductase (130 U/mL) in 0.3 M PBS.
• 7.5 mg phosphorylase A.

During preparation, all chemicals were kept on ice and in subdued light (flavin mononucleotide is light sensitive). When the enzymes and cofactors had been dissolved in the buffer, the pH was set to 7.0. Phosphoglucomutase and phosphorylase A were purchased from Sigma, the other enzymes and NADP were from Roche. All other chemicals were from Sigma.

**Lactate dependent bioluminescence**

To 600 µl of 0.3 M PBS with l-glutamic acid (75 mM), 1,4-dithiothreitol (1.65 mM), flavin mononucleotide (1.3 mM) and capric aldehyde (26 mM) (pH 7.0), a cofactor (NAD) and enzymes were added in the amounts given below:

• 600 µl of 0.5 M NAD dissolved in 0.3 M PBS with 0.3 M HEPES and 0.03 M NaOH.
• 200 µl of lactate dehydrogenase (5500 U/mL) in 0.3 M PBS with 50 mM glutamic acid. To prepare this solution, 1 mL lactate dehydrogenase in 3.2 M ammonium sulfate solution was centrifuged in an eppendorf centrifuge for 1 minute. After centrifugation, the supernatant was removed and the pellet carefully dissolved in 1 mL 0.3 M PBS with 50 mM glutamic acid.
• 200 µl of glutamate-pyruvate transaminase (800 U/mL) in 0.3 M PBS with 50 mM glutamic acid. Glutamate-pyruvate transaminase in PBS with 50 mM glutamic acid was prepared exactly as lactate dehydrogenase in PBS with 50 mM glutamic acid (see above).
• 180 µl of luciferase from photobacterium fischeri (200 mU/mL) in 0.3 M PBS with 50 mM glutamate.
• 200 µl of NADPH-FMN-oxireductase (130 U/mL) in 0.3 M PBS with 50 mM glutamate.

During preparation, all chemicals were kept on ice and in subdued light (flavin mononucleotide is light sensitive). It was important that gloves were used during preparation (as well as during subsequent handling) to avoid contamination of the enzyme solution with lactate from sweat. When the enzymes and cofactors were dissolved in the buffer, the pH was set to 7.0. The enzymes and NAD were from Roche, and other chemicals were from Sigma.

**Standards**

Standards were made by dissolving different concentrations of ATP, glucose, glycogen, and lactate in physiological saline with 8% low molecular weight gelatin. The solution was frozen and sections were made and treated in exactly the same way as the tissue sections. The use of frozen gelatin solutions as calibration standards have been shown to give highly reproducible quantitative data with other enzymatic imaging techniques that are methodologically similar to bioluminescence imaging. Importantly, there was no difference in reaction kinetics between gelatin standards and carotid artery sections (Figure IIA). Furthermore, the bioluminescence intensity was dependent on the total metabolite content of the section and not on the concentration per se. This conclusion could be drawn from an experiment where we changed the ATP content in carotid artery (curve B – tissue concentration 0.6 µmol/g w.w.) and standard sections (curve C – standard concentration 0.6 µmol/g w.w.) by decreasing (to 5 µm = 50%) or increasing (to 20 µm = 200%) the section thickness. The curves were compared with a standard curve made from 10 µm sections (curve A) with different ATP concentrations (0.3 = 50%, 0.6 = 100%, and 1.2 = 200%; the values are in µmol/g w.w.), Figure IIB. The slight deviation from the 10 µm standard curve at 20 µm (both for tissue and gelatin standard) could be due quenching of photons by the thicker section and/or a reduced availability of enzyme solution within the 20 µm section.
Measurement procedure

The set up for bioluminescence registration is schematically displayed in online fig III. A photon counting camera (C2400-47, Hamamatsu Photonics) was mounted underneath an inverted microscope (Axiocvert 135M, Carl Zeiss). A slide with a section (tissue or standard) was dried on a heating plate (95°C) for 10 seconds, placed on the microscope stage and put into focus (1.25x NA 0.035 or 2.5x NA 0.075 Zeiss Plan-Neofluar Optics). A dark field image was registered and then photon counting was initiated in complete darkness at the same time as 5 µL of enzyme solution was applied to the section (Figure III). The resulting images (dark field and bioluminescence) were captured and stored. All measurements were performed at room temperature (23±1°C).

Image analysis

Dark field images were used to outline histological structures in the corresponding bioluminescence images. Standard curves were prepared from mean bioluminescence intensity of standard preparations (Figure 1). The global metabolite concentration was calculated from average bioluminescence within the whole media and metabolite concentration profiles were determined along virtual sections at 3, 6, 9 and 12 o'clock, Figure IVA.

Methodological variation

To obtain a quantitative estimate of methodological variation (Figure IVB), repeated measurements were performed on consecutive 10 µm cryosections (where a virtually identical value would be expected). This variability is likely to include small differences in section thickness between consecutive sections as well as subtle differences in measurement conditions between repeated analyses. Differences within an individual registration (image) were assessed by comparing four analog segments of each artery ring (where again a virtually identical value would be expected). This variation is probably a reflection of differences in section thickness within the section and could also be influenced by small heterogeneities in the application of enzyme solution.

KS 400 software (Carl Zeiss) was used throughout.

Establishment of measurement conditions

The spatial resolution is compromised by diffusion of metabolites from the tissue into the enzyme solution during bioluminescence registration. This results in blurred edges and underestimation of local concentration differences. A similar amount of diffusion was registered for ATP, glucose and lactate while glycogen diffusion was considerably lower (data not shown). Conditions to achieve optimal resolution were defined in an array of experiments and the most important aspects of the results are described below.

Reaction kinetics

The reaction kinetics for the bioluminescence reaction in the different enzyme solutions are shown in Figure V. The peak activity for ATP dependent photon emission occurs immediately whereas for glucose, glycogen, and lactate there is a lag phase of approximately 10-15 seconds (glucose and lactate) or 20-25 seconds (glycogen) before photon emission starts. The peak activity is reached 20 (glucose), 50 (lactate) and 120 (glycogen) seconds later. Thus a short integration time (20 seconds) is sufficient to collect enough photons to obtain an image for ATP while longer integration times are required for glucose (40 seconds), lactate (50 seconds) and glycogen (120 seconds). The longer integration time results in increased substrate diffusion and thus a decreased spatial resolution. Diffusion of glycogen was less pronounced than diffusion of the other metabolites.
Application of enzyme solution

We have described how diffusion may be reduced when the enzyme solution is applied on top of the cryosection when it is already in focus in the inverted microscope. This technique was further developed in the present study, a small black iron holder with 5 µl drop of enzyme solution underneath was placed just above (<1 mm) the frozen section. The iron holder was held in place by an electro magnet that could be turned off to carefully apply the solution to the section exactly at the same time as photon counting was initiated. Strips of adhesive tape at the edges of the holder were used as spacers and ensured that a thin layer of enzyme solution was evenly distributed on top of the section (Figure IIIB). We achieved the best reproducibility between measurements and the highest spatial resolution with this technique.

Temperature and viscosity of the enzyme solution

Other researchers have reduced substrate diffusion by increasing the viscosity of the enzyme solution and/or by lowering the measurement temperature. In our hands, these approaches increased variability within single sections as well as between measurements on consecutive sections. It also decreased the luminescence activity, probably by lowering enzyme activity and/or by reducing the availability of enzyme solution within the tissue, and thus longer integration times were required (>60 seconds). In summary, an increased viscosity or decreased measurement temperature did not increase resolution but it did increase the methodological variation.

Integration conditions and composition of enzyme solutions

To minimize the effect of diffusion, integration time should be kept as short as possible. However, a short integration time means a weak luminescence signal and thus a decreased ability to detect low metabolite concentrations. The luminescence signal can be enhanced by increasing the sensitivity setting of the photon counting system but this increases the number of pixels activated by every photon and thus impairs resolution. Another approach to enhance the luminescence signal is to increase the concentrations of enzymes in the enzyme solutions. However, we found that when enzyme concentrations were too high, the variability between measurements increased. To achieve the best possible results a compromise between these factors was established.

For ATP an integration time of 20 seconds and a sensitivity setting of 8.0 (maximum 10) on the photon counting system gave the best results.

For glucose and lactate longer integration times had to be used because there was a lag phase in the luminescence reaction for these metabolites (Figure V). The luminescence signal could be enhanced by increasing the concentrations of luciferase and Nadp(h)oxireductase in the enzyme solutions for these metabolites. In our experimental set up, the best results were obtained when the concentrations of luciferase and Nadp(h)oxireductase were increased by approximately 50% from the original protocol. This increased the bioluminescence activity (but the lag phase was unchanged) so that a relatively short integration time (40 seconds for glucose and 50 seconds for lactate) could be used. The sensitivity of the photon counting system was set to 9 (maximum 10).

The enzyme solution for glycogen had both the longest lag phase and the lowest peak activity. Thus a long integration time (120 seconds) and a high sensitivity setting, 9.0 (maximum 10), had to be used for the registration. However, glycogen diffusion during registration was relatively minor compared to the other metabolites.

References


Figure I. Composition of enzyme solutions
Biochemical background for the ATP, glucose, glycogen, and lactate dependent bioluminescence reactions utilized to map and quantify metabolite concentrations within cryosections. Enzymes are written in italics. Enzyme abbreviations:
glc-6-P-DH = glucos-6-phosphate-dehydrogenase
LDH = lactate dehydrogenase
GPT = glucose-pyruvate-dehydrogenase

Figure II. Tissue versus standard properties
A. ATP dependent bioluminescence over time in 10 µm sections from carotid arteries (filled rings) and gelatin standards (open rings). The reaction kinetics in gelatin standards and tissue is very similar.
B. Bioluminescence intensity in sections where the ATP content had been altered either by altering concentrations - 0.3 (50%), 0.6 (100%), or 1.2 (200%) µmol/g w.w. - in 10 µm sections (curve A) or by altering section thickness - 5 (50%), 10 (100%), or 20 (200%) µm - in a carotid artery (curve B) and a standard (curve C), both with an ATP concentration of 0.6 µmol/g w.w. (dashed lines). The experiment shows that bioluminescence intensity in 5 µm and 10 µm sections is dependent on the total ATP content within the section and not the concentration per se. At 20 µm, a slight deviation from the 10 µm standard curve is registered, both for the carotid artery and gelatin standard.
Figure III. Measurement set up for bioluminescence imaging
A. An intensified CCD camera (b) is mounted underneath an inverted microscope (a). The camera is connected to a control unit (c) and a monitor (d).
B. Close up of devise used to apply the enzyme solution on the cryosection. 5 µl of enzyme solution is placed underneath a small iron holder which is held in place by an electromagnet. When the electromagnet is turned off, the iron holder is released and the enzyme solution is applied on top of the cryosections. Strips of adhesive tape at the borders of the iron holder ensure that the solution is evenly distributed on top of the section in a reproducible manner.

Figure IV. Analysis of bioluminescence images
A. Global metabolite concentrations were determined by assessing the mean bioluminescence intensities in the media of 5 consecutive cryosections. The tissue borders (*) were identified in darkfield images of the same section. Bioluminescence profiles were determined at 3, 6, 9 and 12 o'clock in each of 3 consecutive bioluminescence registrations and an average profile was calculated. Standard curves (Figure 1) were used to transform the bioluminescence intensities to absolute metabolite concentrations.
B. Variation between repeated measurements on consecutive sections and variation within single sections was determined as shown.
Figure V. The reaction kinetics for the bioluminescence reactions
The peak activity for ATP dependent photon emission occurs within seconds while there is a lag phase before photon emission starts in the other enzyme solutions. The peak activity is reached after 50 (lactate), 100 (glucose), and 120 (glycogen) seconds. The peak photon emission was higher for ATP and glucose than for lactate and glycogen.